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# THE PATHOLOGY OF ARMANNI-EBSTEIN DIABETIC NEPHROPATHY *

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A distinctive glycogenic vacuolization of the cytoplasm of the epithelium of the renal tubules in diabetic patients has long been recognized as a lesion unique in its morphology and in its association with the diabetic state.[1,2] It is considered pathognomonic of diabetes[1,2] and appears related to the severity of the disease and to lack of adequate control.[3] Indeed, with the advent of insulin therapy the lesion has become rare and in the last 25 years has been the subject of but few studies.

Ever since the first descriptions of the lesion by Armanni[4] and Ebstein,[5] conflicting opinions have been expressed concerning the precise localization of the alteration within the affected nephrons and its relationship to renal function in the excretion of glucose. The epithelial vacuolization has been considered to affect principally the loop of Henle in its descending limb[5] or isthmic portion,[6] or both limbs.[7] Although Baehr,[8] in 1913, demonstrated conclusively that the terminal portion of the proximal tubules could also be affected, and Oliver,[8] in a brief reference to a single case, indicated that this was the principal localization of the change, this view has not gained wide currency.

The present study was therefore directed primarily at determining, by three-dimensional visualization of the lesions, their precise localization. On the basis of the findings presented below it now appears that the terminal straight portion of the proximal convoluted tubule is the principal focus of involvement, and that this portion of the nephron varies in its susceptibility to vacuolar change according to its location within the kidney. It seems possible also that the terminal proximal tubule may differ physiologically from those areas situated closer to

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the glomerular headwater, even though morphologic differences between the two zones of the proximal tubule are minor.

## Methods

Specimens of renal tissue from five diabetic patients were selected for study. Selection was based solely on the fact that the diagnosis of diabetes had been clearly established during life, and that routine microscopic sections of the kidneys at necropsy demonstrated the characteristic vacuolization of tubular epithelium in the innermost cortex and outer medulla. Blocks of renal tissue that were available for study had been fixed in either 10 per cent formalin or in Zenker's fluid. Paraffin sections were cut at 4 to 8 $\mu$ and stained with hematoxylin and eosin, HPS (hemalum, phloxine, and saffron), and by McManus' PAS technique.[9] In addition, some sections were prepared by the alcoholic PAS technique for the preservation of glycogen.[10] Confirmation of the presence of glycogen was done by PAS staining of sections initially treated with diastase and with Best's glycogen stain.[11] Additional stains included fat stains on frozen sections using oil red O with hematoxylin counterstain.

Microdissection studies were done in all cases by the hydrochloric acid maceration and dissection procedure as used by Oliver[12] and others.[13,14] Briefly, this method involves careful selection of a piece of formalin-fixed tissue (Zenker fixation renders the tissue too brittle for easy dissection) in such a way as to ensure continuity of cortical nephrons with medullary tubules. After the tissue had been suitably digested in concentrated hydrochloric acid, microdissection was carried out with fine needles under a stereoscopic binocular microscope. Isolated nephrons were picked up on glass slides and protected by coverslips mounted on vaseline runners. Specimens were examined either unstained using a green light source, or colored with Regaud's iron hematoxylin. During the study of individual kidneys several hundreds of nephrons would be observed in the dissecting dish. Many of these were selected for mounting and detailed microscopic study. It should be noted that after acid digestion, iron hematoxylin colors the cytoplasm but leaves nuclei unstained. Details of cell structure are poorly visualized but gross structural distortions are easily observed.

In the course of the investigation it was found that the time of maceration (normally extremely variable) could be accelerated to a few hours by placing the tissue in an incubator at 50° to 56° C. On the other hand, more precisely controlled and uniform maceration was best achieved by carrying out the digestion in the refrigerator at slightly higher than freezing temperatures. Under these conditions beautifully

uniform digestion could be achieved in 10 to 15 days. Permanent preservation of mounted nephrons was accomplished by replacing the aqueous mounting medium with Gelatinol (C). Photographs of mounted specimens were made by serial photomicrography using Ilford Pan F 35 mm. film. Subsequent trimming and montage of the prints allowed accurate reconstruction of the original specimen.

## Summary of Cases

The important clinical features of the five cases are presented in Table I. The oldest patient was a woman of 36 years and three of the five patients were under 17 years. Four were females and all but one of the patients were known to have had diabetes prior to the terminal illness. In each, a complicating illness appeared prior to the terminal coma which developed in all. The complicating illness was an upper respiratory infection in two cases, bilateral otitis media in one, and dental extraction in two cases. In the latter two it appeared that the precipitation of coma was in part referable to infection, and in part to disturbance of normal dietary control resulting from loss of teeth. Impairment of urinary output was noted in one case and anuria in one. More or less

Table I

*Clinical Resumé of Five Cases of Armanni-Ebstein Diabetic Nephropathy*

| Case no. | Age | Sex | Duration of diabetes | Terminal illness | Duration of terminal illness | Blood sugar | | Urine output last 24 hrs. | Coma | Blood non-protein nitrogen |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Highest | Last 24 hrs. | | | |
| | yrs. | | | | | mg. % | mg. % | | | mg. % |
| 1 | 16 | F | 7 days | Bilateral otitis media | 7 days | 945 | 945 | 0 | + | 240 |
| 2 | 8 | F | 5 yrs. | Upper respiratory infection | 2 days | 1,228 | 424 | ? | 48 hrs. | 65.8 |
| 3 | 30 | F | 15 mos. | Extraction of teeth | 11–12 days | 939 | 939 | 205 cc./15 hrs. | 48 hrs. | |
| 4 | 36 | F | 5 yrs. | Extraction of teeth | 2 mos. | 1,390 | | ? | + | |
| 5 | 5 | M | 3 yrs. | Upper respiratory infection | 3 days | 300 | | ? | 24 hrs. | |

marked terminal uremia was noted in two patients. It is evident from the individual protocols that all patients suffered from the complex metabolic derangement that is characteristic of diabetic coma. That is to say that in addition to hyperglycemia and glycosuria, there were varying degrees of dehydration, shock, acidosis and, in case 1, distortion of the serum electrolyte pattern.

## Observations

In all cases there was moderate to marked glycogenic vacuolization of tubular epithelium. In ordinary sections the lesion showed a striking and constant localization astride the corticomedullary junction; but mainly in the outer medulla (Fig. 1). Extension to the innermost cortex was usual, but lesions were never seen in the middle or outer cortex.

The affected epithelial cells were markedly swollen, bulging into and sometimes apparently occluding the lumen (Fig. 2). The cell borders were unusually sharp, perhaps accentuated by virtually total conversion of the cytoplasm into a single large vacuole, in the center of which lay a normal appearing nucleus. Cell borders were sometimes stretched and smooth, at others greatly wrinkled, as though there had been shrinkage of the distended cell (Fig. 2). In the portions of the tubule showing the change, all cells were affected in apparent continuity. Sections stained with either aqueous or alcoholic PAS technique showed high concentrations of globular PAS-positive material in the cytoplasm of the affected cells (Fig. 3). This reaction was prevented by preliminary treatment of the sections with diastase (Fig. 4). Best's reaction for glycogen was also positive in the one case in which alcohol-fixed tissue was available (case 1). Fat stains on kidneys from all five cases, using oil red O on frozen sections, showed no stainable lipid in the glycogen vacuolated epithelium. In cases 2 and 5, however, fine fat droplets were present in the basal portion of the cytoplasm of the non-vacuolated cells of the proximal convoluted tubules.

The affected tubules were usually more or less markedly dilated. There was no noticeable abnormality of the connective tissue or vessels in the vicinity of the lesions. The unaffected tubules did not appear abnormal, i.e., we did not observe gradations or transitions between severely affected and normal tubules. It appeared, therefore, on the basis of conventional microscopic sections that the lesion had probably a rather abrupt beginning and end within the nephron. On the basis of the location of the changes within the renal topography it might have been assumed, as others have done, that the portion of the nephron principally affected was Henle's loop. It was possible, however, in occasional instances where the tubule had been cut longitudinally, to

see toward the cortical aspect an abrupt transition from normal appearing proximal tubular epithelium and the clear distended cells of the lesion (Fig. 2). It was thus apparent that, whatever other areas of the nephron might be affected, the proximal tubule clearly was involved in at least some cases. Dissection studies in all five cases demonstrated this to be true, and showed further that the proximal tubule is invariably the principal site of change (Figs. 5, 6, 7, 8, and 9). More precisely, the area of involvement was the terminal straight portion of the proximal tubule, spilling over occasionally to affect the contiguous beginning portion of the thin limb of Henle's loop (Fig. 7). This terminal portion of the proximal tubule is the relatively straight segment of it that descends into the medulla, that is to say, the relatively nonconvoluted portion of the proximal convoluted tubule. While it might be argued that this indeed is part of Henle's loop, the cells of this zone are morphologically indistinguishable from those of the upper proximal tubule, and are normally considered to be proximal tubule when seen in conventional sections.

The linear portion of the tubule affected was extremely variable, even in nephrons from the same kidney. Sometimes only a very short segment was involved (Fig. 8); at others as much as 20 per cent of the length of the proximal tubule exhibited the alteration (Fig. 7). In the dissected specimens the lesion, in its most fully developed form, appeared as one, or as a series of irregular, fusiform dilatations of the tubule (Fig. 5). These were often eccentric with respect to the axis of the tubule. The lesions were readily visible in the dissecting dish, where, in addition to the obvious dilatation, it was apparent that the tubule was considerably paler and more translucent than the adjacent more normal proximal segments.

Microscopic examination of the dissected specimen usually showed the lesion to consist of a translucent open lacework pattern (Fig. 9), presumably created by the vacuolation and distention of the affected cells. The basement membranes usually appeared intact, although in case 1 where there were also changes of acute nephrosis, foci of necrosis and rupture were apparent (Fig. 10).

In cases where the involvement was discontinuous, the foci of damage formed sausage-like links connected with one another by short segments of normal caliber (Fig. 8). The epithelial cells even between the dilated segments showed vacuolation and pallor. In some instances, and this was true of occasional nephrons from each of the cases, dilatation of the tubule was inconspicuous and pallor of the epithelium was the principal sign of damage.

A curious observation was made in the course of the dissection of

tissue from case 3. Although conventional microscopic sections had shown lesions to be numerous, nephrons from the first block of tissue dissected showed no evidence of damage. In seeking a possible explanation for this discrepancy, it was realized that the nephrons that arise close to the capsule of the kidney have Henle's loops that often do not reach or penetrate the medulla. It was postulated that if these nephrons did develop Armanni-Ebstein lesions, such lesions should occasionally be seen in the central or outer cortex in conventional sections. Review of the sections from all five cases showed that in none of them were lesions present in these areas. From this it was reasoned that the exclusively cortical nephrons were probably either less susceptible to glycogenic vacuolization or perhaps entirely immune. Dissection of additional blocks of kidney from case 1 with special attention to this possibility revealed that lesions were numerous and extensive in nephrons that penetrated the medulla but rare in those that did not. Further confirmation could not be made in the remaining cases since no more tissue was available for dissection. On the basis of the distribution of lesions in sections from these cases, however, it is probable that the exclusively cortical nephrons in them, too, were relatively resistant to damage. It should be pointed out also that the "immune" nephrons, in addition to their cortical location, were usually ones whose loop of Henle was short, and in which the thin segment was either very short, or non-existent (Fig. 11).

## Discussion

There are several other forms of vacuolar nephropathy from which it is not difficult to distinguish the Armanni-Ebstein lesion. In poisonings from diethylene glycol or dioxane the change affects the entire proximal tubule and is usually associated with hyaline droplet formation or necrosis of cells in patchy fashion.[2] The vacuolation in patients treated with hypertonic sucrose or dextran is much finer and again affects the entire proximal tubule.[15] The changes associated with potassium loss in severe diarrhea affect isolated cells in random fashion.[16]

The epithelial changes in cases of von Gierke's disease are similar to those in diabetic cases in their appearance[17] but differ in that the upper portion of the proximal tubule appears selectively affected. The evidence that in some cases of von Gierke's disease there is impairment of intracellular glycogenolysis[18] might, however, suggest a similar explanation in the kidneys of diabetic patients. Fatty vacuolization as is seen in potassium deficient rats[19] is generally basal, and of course the

fatty nature of the vacuoles is demonstrated by appropriate stains. Similar fatty change was found in some of our cases, but in all instances the upper proximal tubule was affected and not the portion involved by Armanni-Ebstein lesion.

The latter lesion is distinctive in its nephronal localization, and this is reflected in the distribution of the changes as seen in conventional sections, astride the corticomedullary junction.

The obvious association of the Armanni-Ebstein change with the state of uncontrolled diabetes suggests almost too readily an association between the renal alteration and the high level of blood sugar and of urinary glucose excretion. Robbins[20] related the appearance of the lesions in alloxan-diabetic rats to blood glucose levels of at least 350 mg. per cent for a duration of 5 days or more. In spite of this evidence, it is well to remember that the renal metabolic disturbance in human patients with this lesion is a complex one in which there is usually distortion of electrolyte and fluid distribution and that one or all of these solute changes may be responsible for the type of alteration observed, rather than glucose alone.

Whatever the contribution by the factors indicated above, it is clear that glycogen and glucose are fundamental in the development of the renal changes. The exact means by which glycogen accumulates in the renal epithelium remains unclear. It is possible that the affected portion of the tubule, normally exposed to little or no filtered glucose,[21] is less well equipped to handle this substance than are the more proximal areas. The cells in the affected segment of the nephron, though normally identical with their more proximal neighbors, may possess a greater capacity for glycogenesis than for glycogenolysis when faced with excessive concentrations of glucose substrate. Another possibility would be that excessive glycogenesis, or defective glycogenolysis, results in some way from the effects of acidosis or dehydration, rather than reflecting a simple response of a normal physiologic mechanism to uncomplicated substrate excess.

The most impressive finding in this study was the relative immunity to damage of those nephrons that did not penetrate the corticomedullary junction. This was in marked contrast to the almost general susceptibility of the penetrating nephrons. Although a number of possible explanations can be proposed for the different susceptibility of these two classes of nephrons, there is no direct evidence on this point in the material at hand. An obvious morphologic difference between the affected and uninvolved nephrons is that the latter have a short loop and a non-existent or rudimentary thin limb. On this basis it might be pro-

posed that the thin limb normally imposes a degree of stasis just above its commencement and that this plays a rôle in the pathogenesis of the lesion.

Resistance of the cortical nephrons to glycogenic change may, on the other hand, be related to the fact that their loops lie in a more richly vascular environment than do those of the penetrating nephrons. This could lead to improved glucose transfer with reduced liability to intracellular stasis in the form of glycogen. It would seem more probable that this is the case than that this minority of nephrons is functionally different from the rest, although the latter possibility cannot be excluded.

Whatever the reason for the resistance to glycogenic change demonstrated by the cortical nephrons, it would be of some interest to determine whether they are also resistant to other forms of damage, e.g., poisoning, acute nephrosis.[22]

## Summary

The kidneys from five cases of Armanni-Ebstein diabetic nephropathy were studied by observation of conventional microscopic sections and by the technique of maceration and dissection of individual nephrons. The lesions in all cases consisted of marked glycogenic vacuolization of the epithelium of renal tubules in the outer medulla and innermost cortex. Tubules in the central and outer cortex were not affected.

Dissection of nephrons showed the lesions to be localized to the terminal straight segment of the proximal convoluted tubule, with occasional extension into the contiguous portion of the thin limb. It was established in one case, and inferred in the others, that the exclusively cortical nephrons, which have a short Henle's loop and which do not penetrate the medulla, are relatively resistant to glycogenic vacuolization. The possible reasons for this reduced susceptibility to damage are considered.

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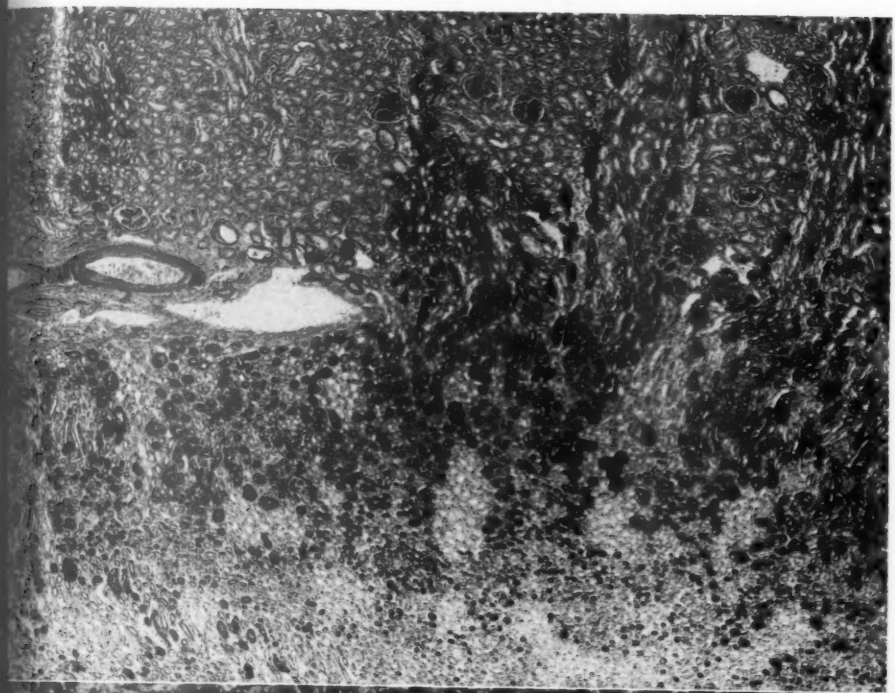
[ *Illustrations follow* ]

## LEGENDS FOR FIGURES

FIG. 1. Case 3. Corticomedullary junction of kidney. Dark glycogen deposits are concentrated mainly in tubules of outer medulla with some irregular involvement of innermost zone of cortex. Periodic acid-Schiff (PAS) stain with hematoxylin counterstain. × 25.

FIG. 2. Case 1. Dilatation and vacuolization of tubules. Partial involvement of a proximal tubule is seen at the center of the photograph. Other tubules show involvement of all epithelial cells. Hematoxylin and eosin stain. × 200.

# WARNING



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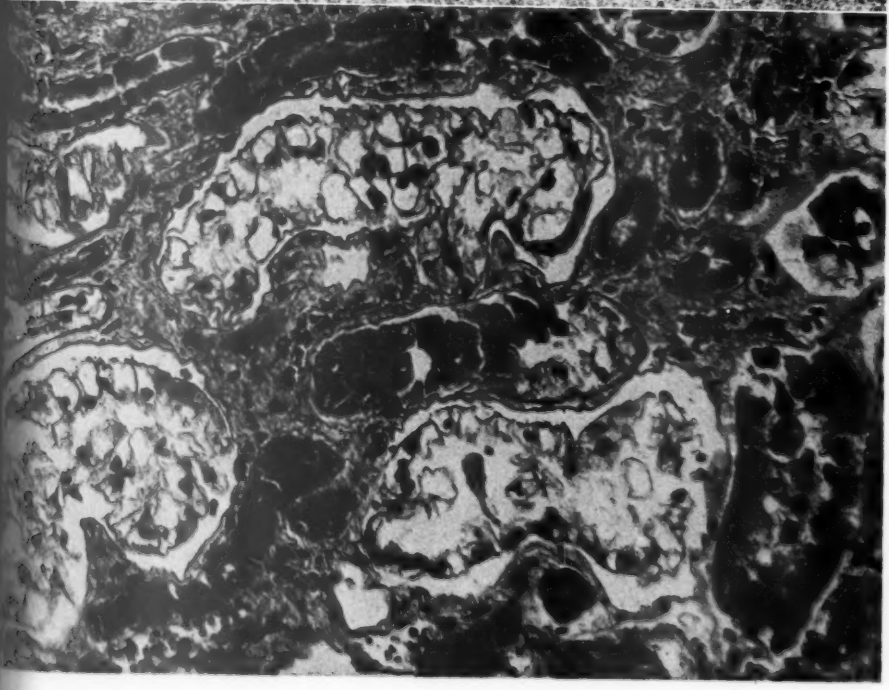

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FIG. 3. Case 2. PAS-positive material in epithelial cytoplasm of involved tubules. PAS stain with hematoxylin counterstain. × 160.

FIG. 4. Case 2. PAS positivity removed from vacuolated cells by preliminary treatment with diastase. Diastase digestion followed by PAS reaction. × 200.

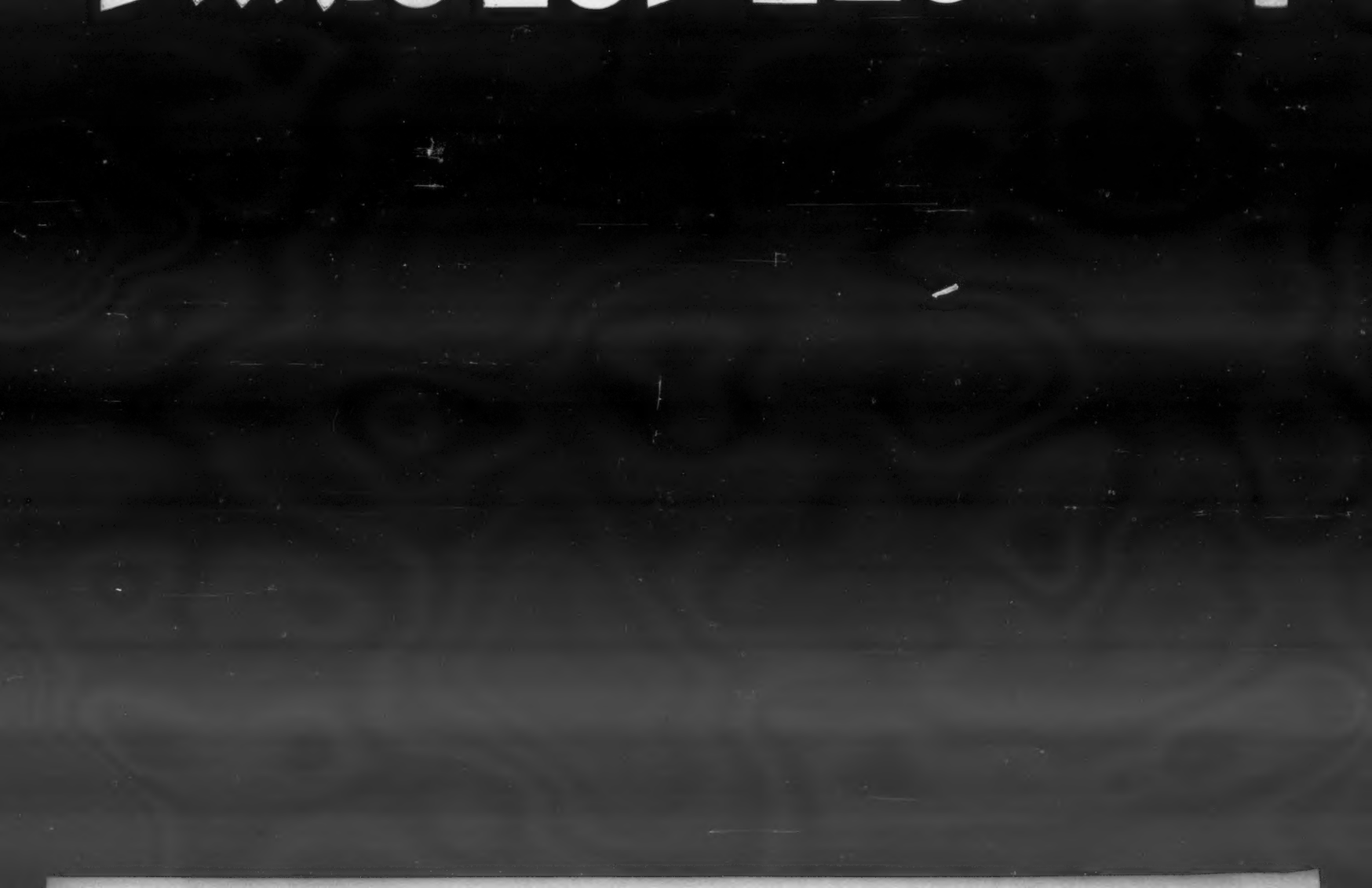

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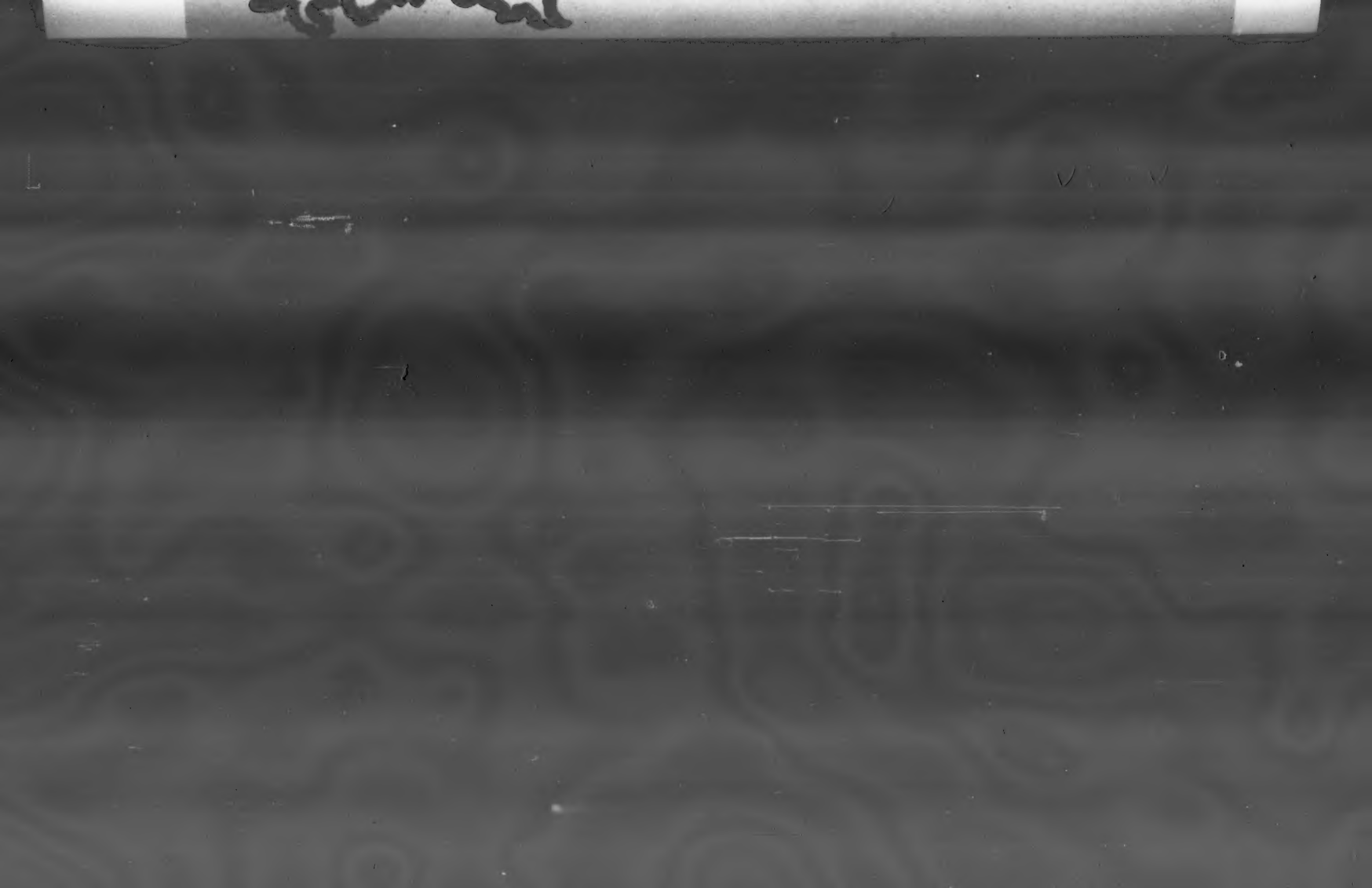
FIG. 5. Case 2. Photomontage reconstruction of isolated glomerulus, proximal tubule, and beginning of Henle's loop. The glomerulus and most of the proximal tubule are normal. At the distal end of the specimen there is irregular fusiform dilatation, pallor and translucency of the terminal proximal tubule, and possibly of the commencement of the thin limb. The remainder of the thin limb is normal in appearance. Unstained aqueous mount. × 40.

FIG. 6. Case 5. Isolated upper nephron showing epithelial pallor and dilatation of terminal proximal tubule. The mosaic appearance was produced by vacuolation of epithelium. Unstained aqueous mount. × 40.



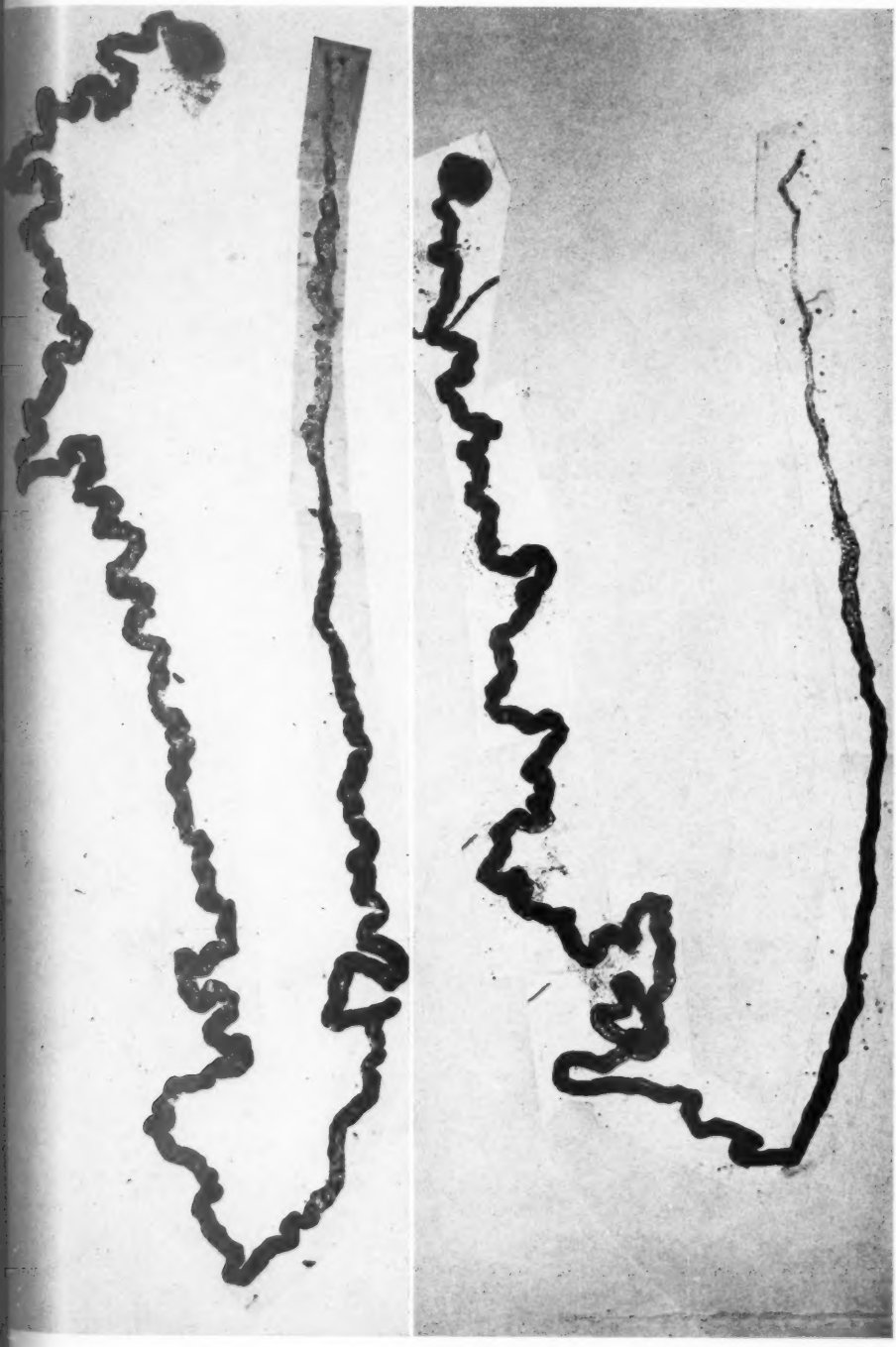


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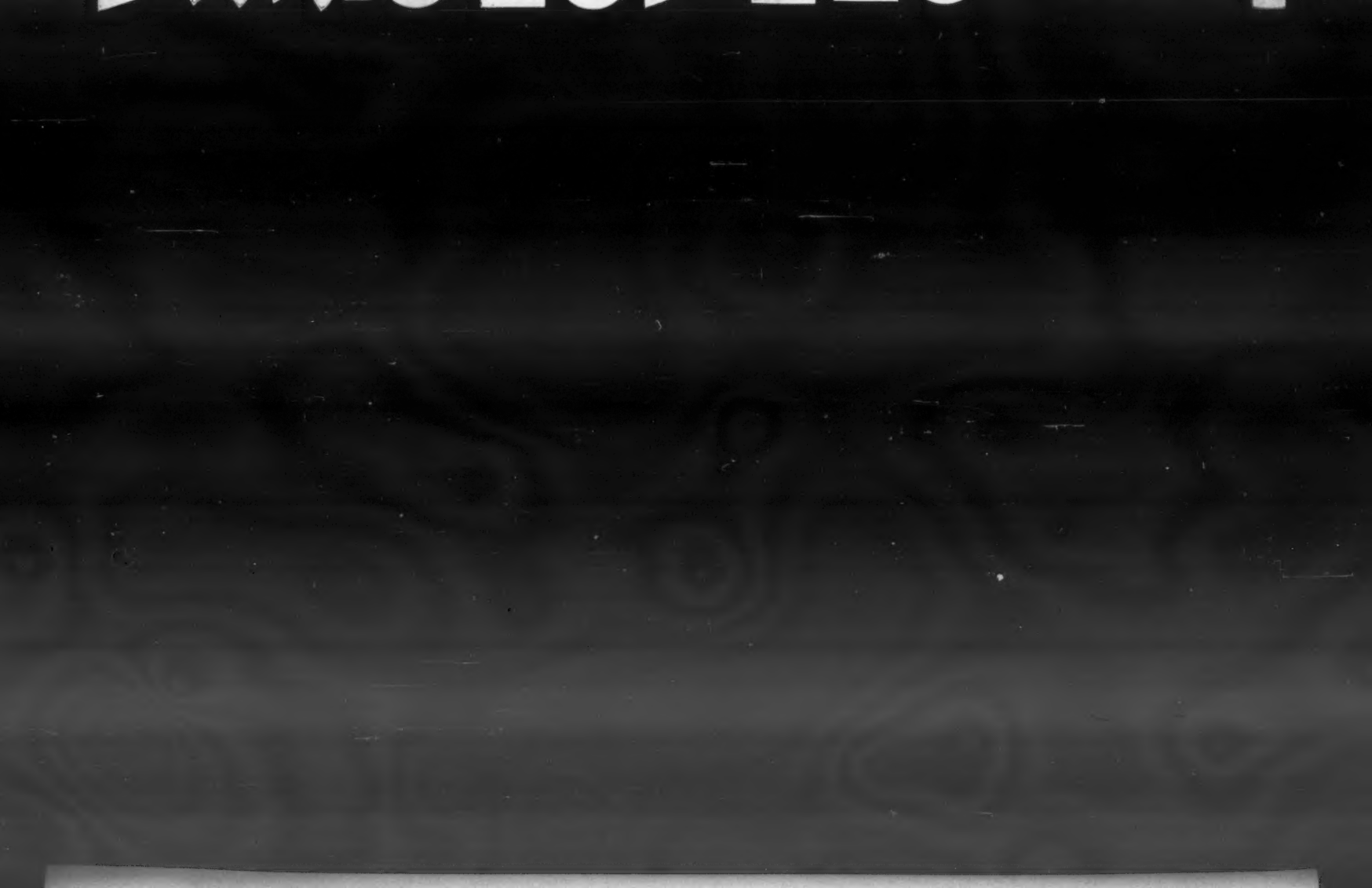
FIG. 7. Case 3. Terminal portions of proximal tubule showing irregular saccular dilatation. Unstained aqueous mount, × 40; enlarged area, × 160.



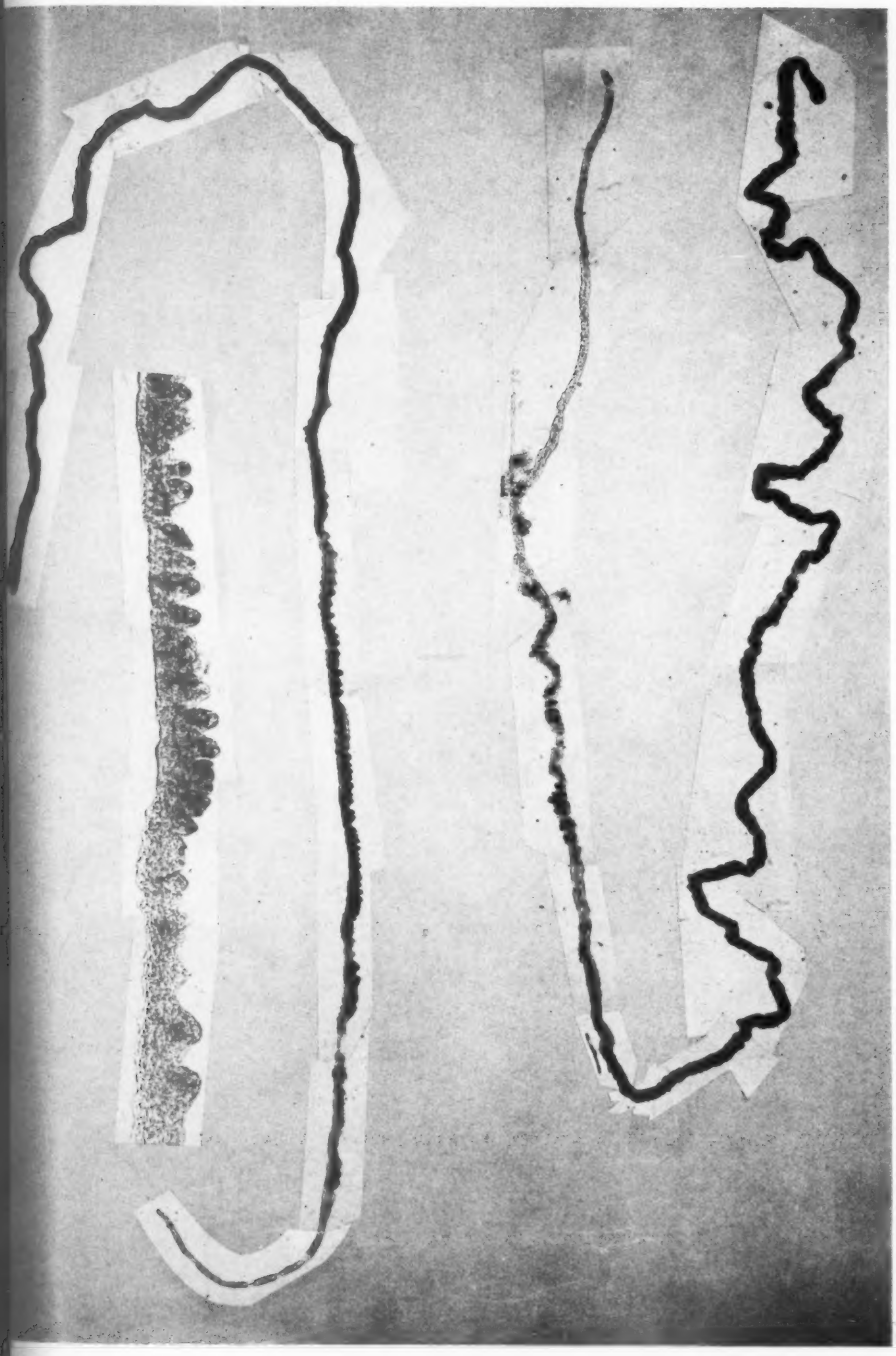


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FIG. 8. Case 1. Small localized saccular lesions confined to the thin limb. Unstained aqueous mount. × 40.

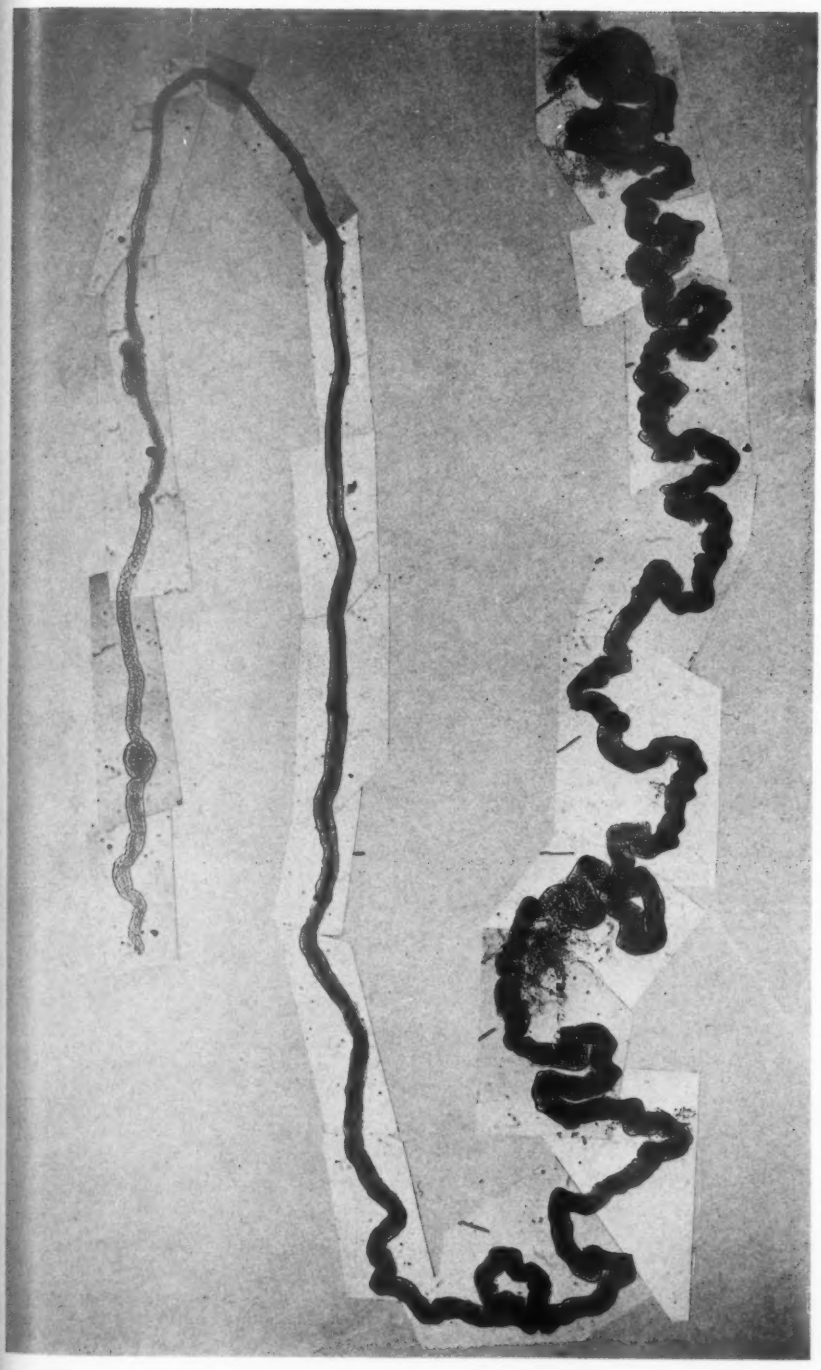

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FIG. 9. Case 4. Diffuse Armanni-Ebstein lesion of terminal proximal tubule. High-power photograph shows complex refractile feltwork appearance produced by distended cells. Unstained aqueous mount, × 40; high power, × 165.

FIG. 10. Case 1. Two distal convoluted tubules and collecting tubule. Patient died in renal failure. Specimen shows irregular foci of necrosis of tubule, obstruction by casts. Adhesive strands of connective tissue are attached to the left tubule. Iron hematoxylin Gelatinol (C) mount, × 40; high power, × 160.

# MEXICO

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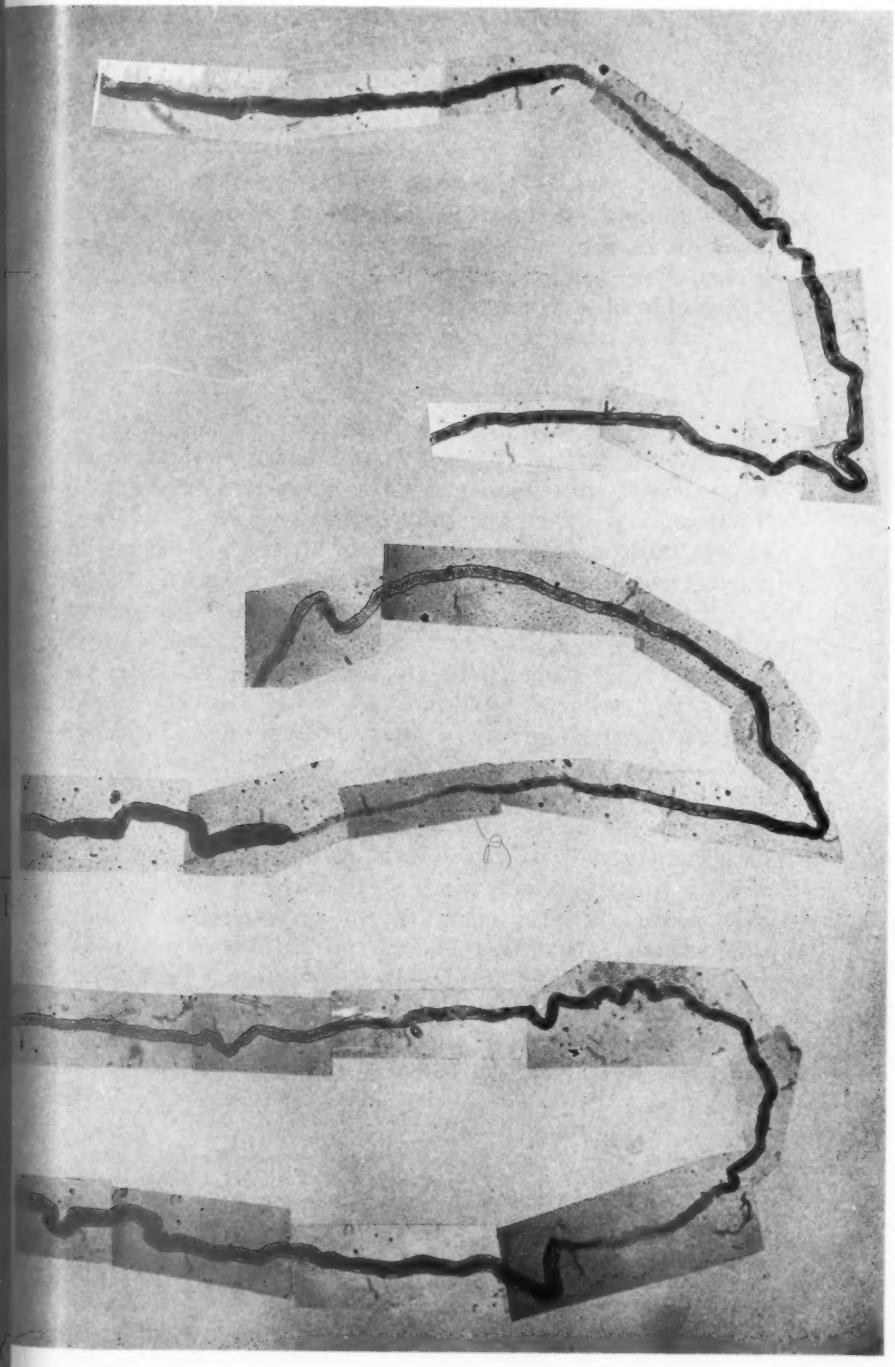
FIG. 11. Case 1. Three uninvolved loops from nephrons originating just beneath the renal capsule. A short thin limb is present in the center specimen only. Unstained aqueous mounts. × 40.



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# EXPERIMENTAL CARCINOMA OF THE UTERINE CERVIX IN THE MOUSE

## A Gross and Histopathologic Study *

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Although squamous cell carcinoma of the uterine cervix has been produced experimentally in mice by numerous investigators,[1-4] the detailed pathologic anatomy and histology of these tumors have not been described. This report deals with a gross and microscopic study of 139 experimental tumors of the uterine cervix induced in the laboratories of the Ohio State University during the past 4 years.

### Materials and Methods

Two hundred and sixty-one $C_3H$ female mice, weighing between 18 and 22 gm., were treated intravaginally with either 3,4-benzpyrene or 20-methylcholanthrene during the course of various experiments at our institution. One hundred and thirty-nine mice (53.2 per cent) developed carcinoma of the uterine cervix or vagina. Series 1, 2, and 3 (1953–55) consisted of 226 mice, which were painted twice weekly intravaginally with a 1 per cent solution of 3,4-benzpyrene in acetone. The technique of painting is described in detail in a previous report.[8] In series 4 and 5 (1955–56), comprising a group of 35 mice, a string impregnated with either 3,4-benzpyrene or 20-methylcholanthrene crystals was inserted and suspended in the endocervical canal by the Murphy technique.[2] Vaginal smears were prepared twice weekly and stained by the method of Papanicolaou.[5] When cytologic evidence of carcinoma was observed, the animals were sacrificed at various periods so that both early and far advanced lesions could be studied. The uterus and vagina were removed; if there was gross evidence of extension of tumor or metastases, the involved lymph nodes and organs were removed also, fixed in 10 per cent buffered formalin, and embedded in paraffin. Serial sections were stained with hematoxylin and eosin and examined microscopically. Selected sections from each tumor were stained by the Regaud modification of Heidenhain's iron hematoxylin method[6] for demonstration of tonofibrils. The tumors were classified by the criteria suggested by Martzloff.[7]

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† National Science Foundation Postdoctoral Fellow in Pathology.

## Results

### *Incidence and Distribution of Lesions*

The experimental induction of carcinoma of the uterine cervix and vagina in mice by various methods of application of carcinogens is summarized in Table I.

Table I

*Experimental Induction of Carcinoma of the Uterine Cervix and Vagina in C3H Mice*

| Experiment | Method | No. of mice | Non-invasive carcinoma | Invasive carcinoma | Total | Percentage | Weeks of development |
|---|---|---|---|---|---|---|---|
| 1st Series 1953 | P-BP | 60 | 19 | 12 | 31 | 51.6 | 22–54 |
| 2nd Series 1954 | P-BP | 80 | 21 | 15 | 36 | 45.0 | 16–40 |
| 3rd Series 1955 | P-BP | 86 | 17 | 27 | 44 | 51.2 | 24–53 |
| 4th Series 1955 | S-BP | 15 | 3 | 8 | 11 | 73.3 | 12–38 |
| 5th Series 1956 | S-MC | 20 | 7 | 10 | 17 | 85.0 | 11–33 |
| | | 261 | 67 | 72 | 139 | 53.2 | |

P-BP = painted with a 1 per cent solution of 3,4-benzpyrene in acetone.
S-BP = treated with 3,4-benzpyrene-impregnated endocervical string.
S-MC = treated with 20-methylcholanthrene-impregnated endocervical string.

A considerable variation in the incidence of tumors and the latent period of induction was observed in the five series. The percentage of animals which developed carcinoma varied from 45 per cent for those painted with carcinogen (series 1, 2, and 3) to 73 and 85 per cent for those in which an endocervical carcinogen-impregnated string was employed (series 4 and 5). The latent period of induction, which ranged from 22 to 54 weeks in the painted series, was shortened to 11 to 33 weeks when carcinogen-impregnated strings were used.

Malignant lesions restricted to the uterine cervix were found in 57 animals, lesions involving both the uterine cervix and vagina were present in 55 animals, and lesions limited to the vaginal mucosa were seen in 27 animals (Table II).

In the series in which an endocervical string was used, a greater percentage of tumors was localized solely in the uterine cervix, in contrast to the considerable number of vaginal carcinomas and lesions involving both sites when tumors were induced by painting (series 1, 2, and 3). Animals in which either separate carcinomas were found in the uterine cervix and vagina or in which the lesions were so extensive that the primary sites were not ascertainable were interpreted as having developed carcinoma at both sites.

### *Pathologic Anatomy*

The majority of invasive tumors were of the exophytic type and varied considerably in size. The largest tumor measured 2.7 by 2.2 by 2.4 cm. (Fig. 1), while the smallest lesion was demonstrable only under the microscope. Grossly early lesions appeared as reddish, granular,

TABLE II

*Sites of Neoplasia*

| Series | Vagina | Cervix | Both | Total |
|---|---|---|---|---|
| 1 | 8 | 11 | 12 | 31 |
| 2 | 9 | 11 | 16 | 36 |
| 3 | 9 | 13 | 22 | 44 |
| 4 | 1 | 8 | 2 | 11 |
| 5 | 0 | 14 | 3 | 17 |
| Total | 27 | 57 | 55 | 139 |

TABLE III

*Sites of Tumor Extension and Metastases*

| Site | No. of mice |
|---|---|
| Pelvic lymph nodes | 32 |
| Lateral pelvic wall | 28 |
| Bladder | 22 |
| Ureters | 19 |
| Rectum | 15 |
| Liver | 3 |
| Lung | 2 |

papillary growths which bled readily when touched or gently rubbed with a cotton-tipped applicator. Most of the cervical lesions were located near the external os at the portio vaginalis (Fig. 2), or high on the lateral cervical walls. No lesions were found at the squamo-columnar junction. Early vaginal lesions were located in two areas: either low near the vaginal orifice or high at the vaginal fornices. In advanced lesions the vagina was filled with a large mass which surrounded the uterine cervix, showed foci of ulceration and hemorrhage, and obliterated the vaginal fornices (Fig. 3). All animals with advanced lesions showed evidence of vaginal bleeding. On section many of these tumors showed large, ragged cavities filled with necrotic and infected tumor tissue.

A considerable number of invasive carcinomas showed extension into the pelvic lymph nodes (Fig. 4), lateral pelvic wall, broad ligaments, urinary bladder, and rectum (Fig. 5) in a manner strikingly similar to that seen in human cervical cancer (Table III). Ureteral obstruction (Fig. 6) with accompanying hydronephrosis and pyelonephritis was seen in 19 animals. Gross and microscopic evidence of metastases to lung or liver was present in five instances.

### *Histopathologic Findings*

Both intra-epithelial, or non-invasive, and invasive carcinomas were observed as shown in Table IV. Microscopically, non-invasive carcinomas of two distinct morphologic types were distinguishable: diffuse

and focal. Sixty-three animals (94.1 per cent) showed intra-epithelial lesions of the diffuse type (Fig. 7) with a marked diffuse papillomatous proliferation of basal and parabasal cells within widened epithelial boundaries and with intact basement membranes. The cells were predominantly of intermediate size although some variation was observed.

TABLE IV
*Histologic Types of Carcinoma*

| | Vagina | Cervix | Both | Total | Percentage |
|---|---|---|---|---|---|
| Non-invasive carcinoma | | | | | |
| Diffuse type | 15 | 25 | 23 | 63 | 94.1 |
| Focal type | 1 | 2 | 1 | 4 | 5.9 |
| | | | | 67 | 100.0 |
| Invasive carcinoma | | | | | |
| Spinal cell type | 4 | 7 | 10 | 21 | 29.2 |
| Transitional cell type | 6 | 17 | 13 | 36 | 50.0 |
| Spindle cell type | 1 | 5 | 8 | 14 | 19.4 |
| Muco-epidermoid type | | 1 | | 1 | 1.4 |
| | | | | 72 | 100.0 |

In addition there were nuclear enlargement, hyperchromasia, and numerous mitotic figures, many of which were atypical. Considerable disturbance and, in some instances, complete absence of normal stratification and differentiation of superficial epithelial cells were observed. In 4 animals (5.9 per cent) intra-epithelial lesions of the focal type were observed in low, atrophic appearing epithelium with discrete areas of papillomatous basal cell growth showing small foci of malignant change (Fig. 8). The most outstanding feature of this lesion was the absence of epithelial cell overgrowth and crowding so characteristic of the diffuse type. No evidence of transition from focal to diffuse lesions was seen. Multicentric areas of non-invasive malignant transformation were observed in 21 animals.

Invasive carcinomas showed a disruption of the basement membrane with sheets or islands of malignant squamous cells invading the underlying stroma (Fig. 9). Invasive lesions varied in size from wide fields of malignant transformation to small microscopic lesions (microcarcinomas) which were largely limited to the cervical mucosa but showed morphologic evidence of early invasion as shown in Figure 10. In 18 animals various stages of progressive malignant transformation were present simultaneously. Multicentric lesions were demonstrable in 33 animals.

Although the majority of invasive tumors induced were of the squamous cell type, marked cytologic variation was observed. On this

basis four distinctly different cytologic variants could be differentiated: well differentiated (spinal cell) type, moderately well differentiated (transitional cell) type, undifferentiated (spindle cell) type, and muco-epidermoid type.

The cytologic characteristics of spinal cells as defined by Martzloff[7] consist of polyhedral cells with ample eosinophilic cytoplasm and well defined cell membranes (Fig. 11). The nuclei are moderately hyperchromatic and occasionally show macronucleoli. In addition, we observed well developed tonofibrils in 73 per cent of the epidermoid tumors of the spinal cell type. There was also a tendency for the cells to form wide sheets. Keratinization with epithelial pearl formation was prominent, while mitotic figures were scarce. These well differentiated cells closely resembled the normal cells of the upper stratum spinosum of cervical epithelium in the mouse during estrus. Tumors consisting predominantly of well differentiated spinal cells were found in 21 animals (29.2 per cent), as shown in Table IV.

Transitional cells were more rounded and showed a decrease in the amount of cytoplasm, with large hyperchromatic nuclei, frequent macronucleoli, and mitotic figures (Fig. 12). Tonofibrils were demonstrable in 22 per cent of the squamous cell carcinomas of the transitional cell type. Keratinization and epithelial pearl formation were seen only occasionally. Foci of necrosis were more frequent than in the spinal cell group. These cells resemble those found in the lower portions of the stratum spinosum of cervical epithelium during estrus. In three instances small foci of malignant squamous cells which appeared to be secreting mucus were found interspersed among sheets of tumor cells. The mucus was PAS-positive and not salivary-amylase labile. Tumors of the moderately well differentiated transitional cell variety were observed in 36 animals (50 per cent).

Spindle cells appeared as elongated cells with scant, pale, eosinophilic cytoplasm, large hyperchromatic nuclei, and often multiple macronucleoli (Fig. 13). Numerous mitotic figures were present also. Keratinization and epithelial pearl formation were absent. Although tonofibrils were absent, these cells resembled the spindle-shaped hyperchromatic cells contained in the germinal layer of cervical epithelium. Necrosis with secondary bacterial infection was a common finding. Malignant lesions consisting primarily of undifferentiated spindle-shaped cells were present in 14 animals (19.4 per cent).

In a single instance (1.4 per cent) an invasive muco-epidermoid tumor was observed. Morphologically, it was characterized by nests of moderately well differentiated squamous cells among which were interspersed many acinus-like structures. These were lined by squamous

cells which contained varying amounts of pale blue staining cytoplasmic material and showed transition to plump, rounded cells filled with mucus (Fig. 14) which was PAS-positive and resistant to salivary-amylase digestion. Epithelial pearl formation was absent.

## Discussion

Experimentally induced carcinoma of the uterine cervix and vagina in $C_3H$ mice showed a striking biologic and pathologic resemblance to human cervical cancer. Invasive lesions extended locally into surrounding tissues, metastasized to pelvic lymph nodes, produced obstructive uropathy, and in several instances metastasized widely. The experimental lesions differed from their human counterparts only in their anatomical distribution. The complete absence of lesions at the squamocolumnar junction in the mouse may be due to its considerable distance from the portio vaginalis. The endocervix in the mouse is lined by squamous epithelium with epithelial transition occurring at the bifurcation of the uterus. Thus neither vaginal nor low endocervical application of carcinogen would reach this area. The shortened latent period and higher yield of tumors following the endocervical application of carcinogen-impregnated strings is probably the result of higher concentrations of carcinogen in continuous contact with the epithelium.

Non-invasive and early invasive lesions showed a tendency to separate from the underlying stroma in a manner similar to that reported in human cases of non-invasive cancer by Reagan *et al.*[8] This may be due to the progressive loss of tonofibrils during carcinogenesis as shown by von Albertini.[9] However, the demonstration of tonofibrils in well differentiated tumors suggests a relation between cellular differentiation and tonofibril formation.

The presence of small foci of malignant basal cells localized solely in the stratum germinativum in the focal type of non-invasive carcinoma strongly suggests that at least in some instances the neoplastic change may originate there. Furthermore, the multiple foci and extensive diffuse fields of cervical mucosa which showed malignant changes are evidence in favor of a multicentric pattern of origin. This finding is in accord with the view held by Willis[10] that the carcinogenic stimulus acts on large groups of cells and extensive areas of epithelium rather than on a single cell.

The cytologic variants of squamous cell carcinoma, as Martzloff[7] pointed out, closely resemble the cells of the various layers in mature squamous epithelium and represent varying degrees of cellular differentiation.

It is interesting that a muco-epidermoid tumor and mucus-secreting foci should be observed in tumors arising from squamous epithelium devoid of mucus-secreting cells. However, since it has been well established that squamous epithelium of the genital tract in mice has the potential of mucus production and is capable of mucification under a variety of physiologic and hormonal stimuli,[11,12] mucus production in malignant squamous cells must be interpreted as a metaplastic reaction.

In view of the ease with which cancer of the uterine cervix can be induced in mice and its similarity to analogous lesions in man, it is probable that experimental cervical tumors may prove of value in biologic and therapeutic studies.

## Summary

Attempts at the experimental induction of carcinoma of the uterine cervix in 261 C3H mice resulted in 139 malignant lesions (53.2 per cent). Non-invasive carcinoma was observed in 67 animals, while 72 mice developed invasive carcinoma. Invasive lesions showed intrapelvic extension, metastases, and obstructive uropathy in a manner similar to that observed in analogous tumors in man.

Histopathologic studies showed the majority of lesions to consist of moderately well differentiated squamous cells. In 14 animals (19.4 per cent) poorly differentiated squamous cell carcinoma was observed, and in a single animal a muco-epidermoid carcinoma was seen.

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## LEGENDS FOR FIGURES

All photomicrographs were made from sections stained with hematoxylin and eosin.

FIG. 1. Mouse with the largest tumor induced, 50 weeks after painting was started.

FIG. 2. Epidermoid carcinoma of papillary exophytic type near the external os at the portio vaginalis. × 55.

FIG. 3. Advanced cervical epidermoid carcinoma filling the vagina and showing focal ulceration.

FIG. 4. Pelvic lymph node containing metastatic squamous cell carcinoma. × 135.

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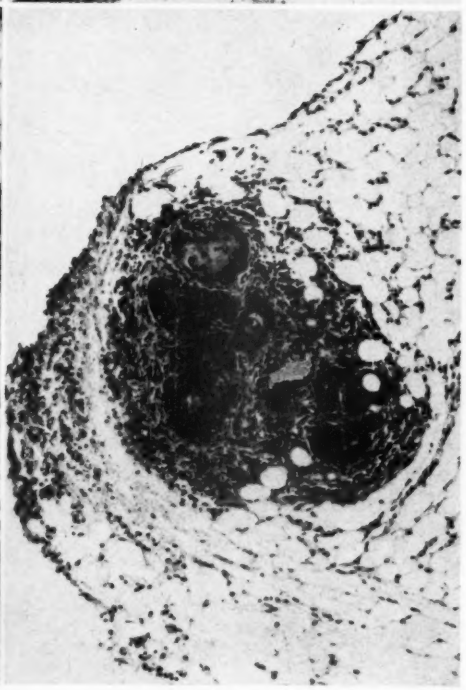

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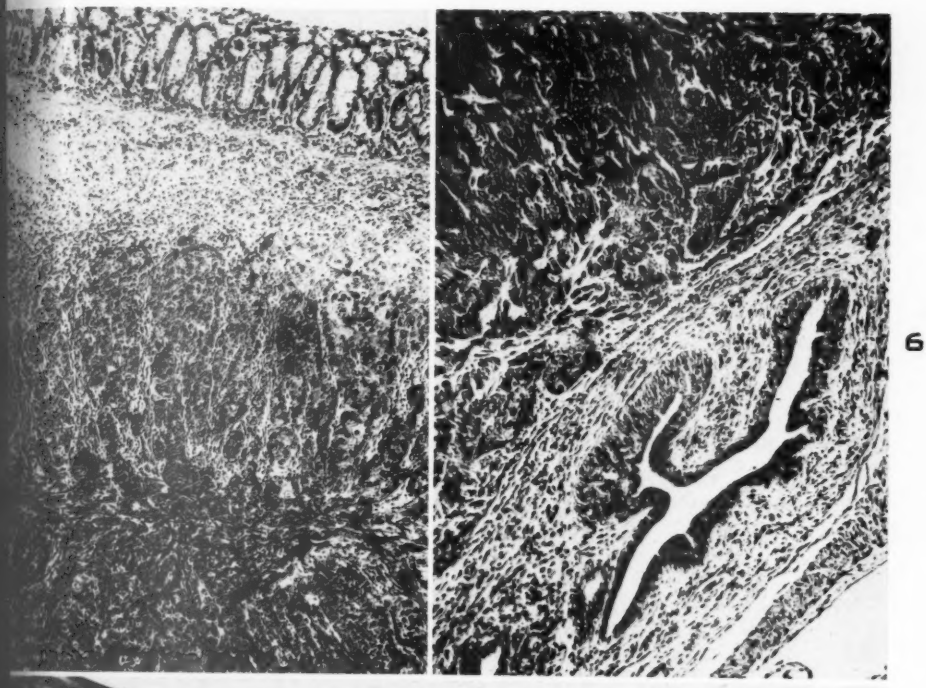
FIG. 5. Rectum showing tumor invasion of the muscularis and submucosa. × 80.

FIG. 6. Periureteral extension of tumor with compression. × 90.

FIG. 7. Intra-epithelial carcinoma showing a marked, diffuse, papillomatous proliferation of basal and parabasal cells with cellular crowding, hyperchromasia, and mitotic figures. The separation of epithelium from the stroma may be noted. × 450.



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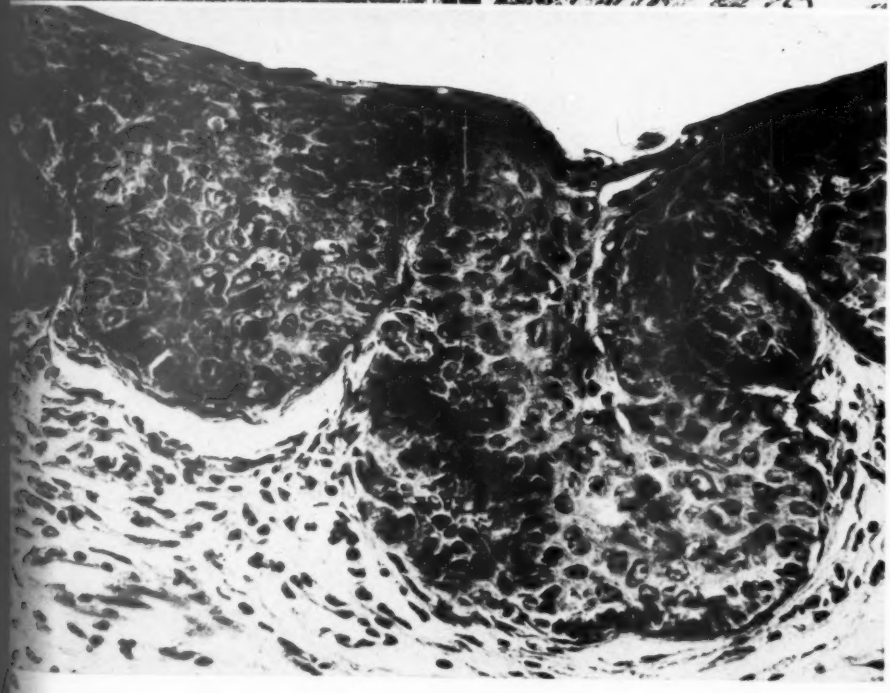

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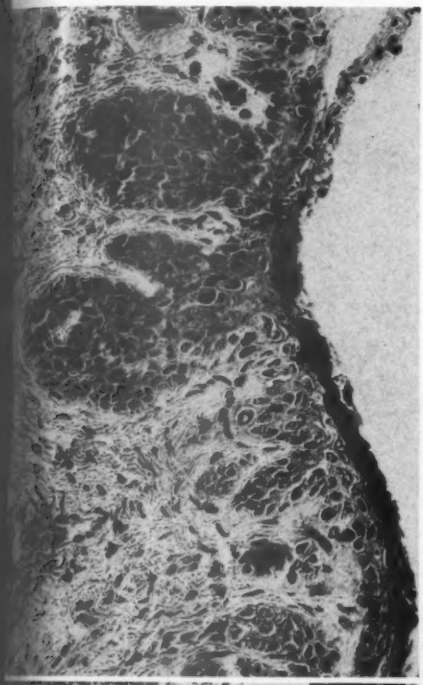
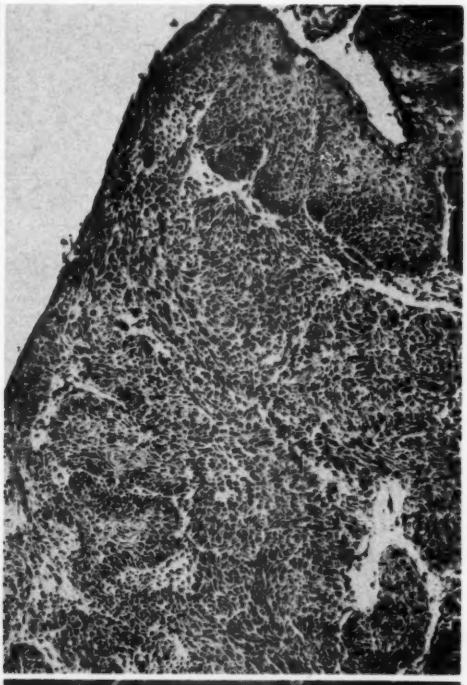
FIG. 8. Intra-epithelial carcinoma showing small foci of malignant basal cells. × 325.

FIG. 9. Invasive carcinoma of the uterine cervix, showing disruption of the basement membrane with sheets of malignant cells invading the stroma. × 115.

FIG. 10. Microcarcinoma largely limited to the cervical mucosa and showing early invasion. × 220.

FIG. 11. Well differentiated epidermoid carcinoma showing cells of the spinal type with small nuclei, ample cytoplasm, and well defined cell membranes. × 260.





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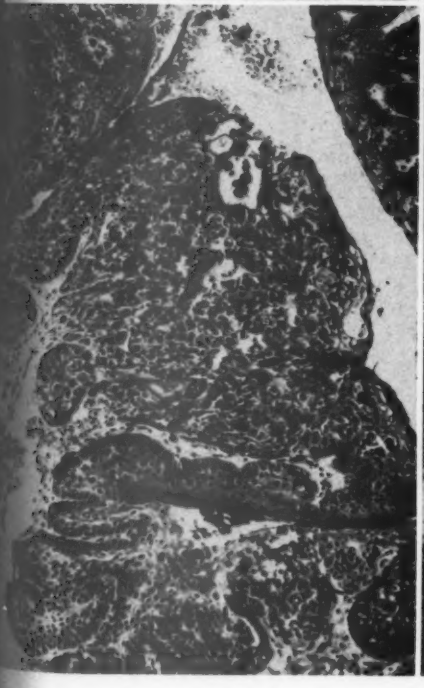

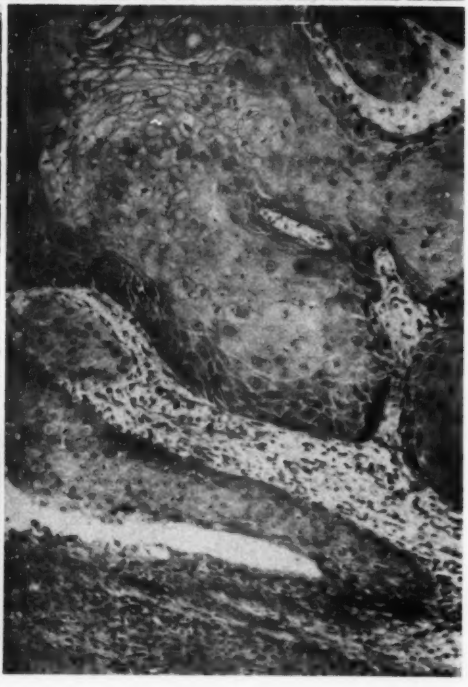

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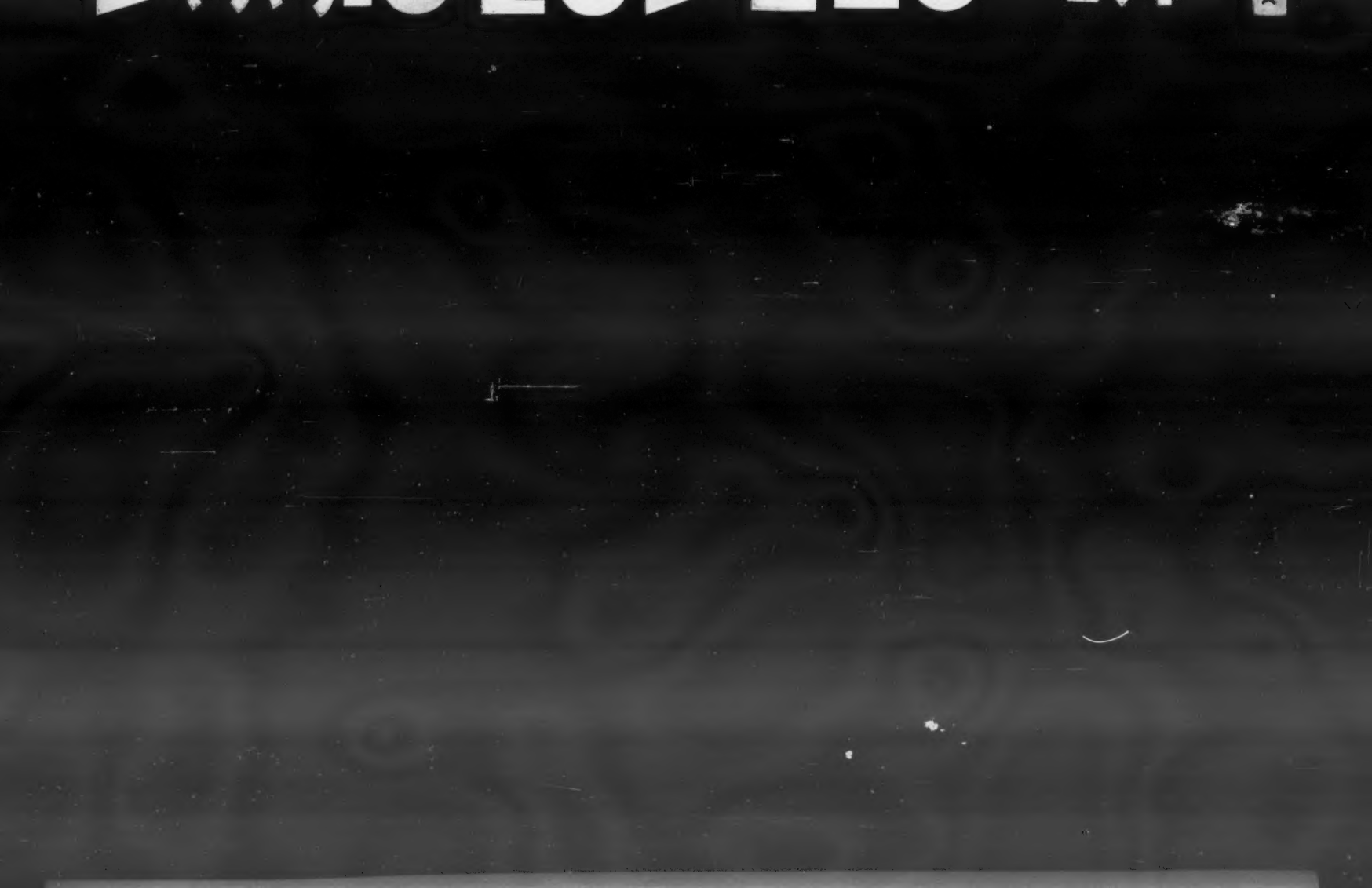
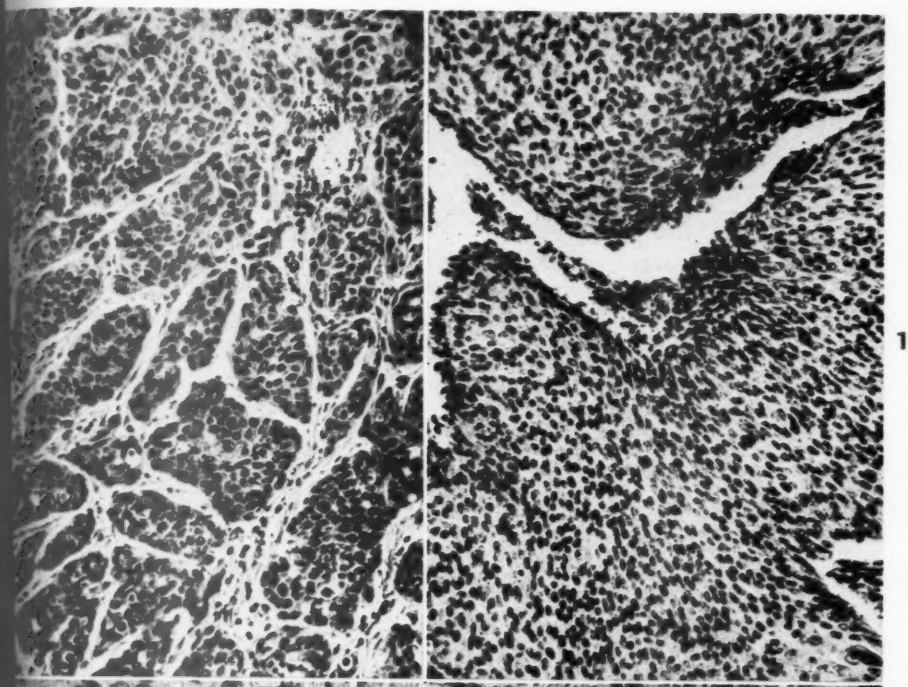
FIG. 12. Moderately well differentiated epidermoid carcinoma of transitional cell type showing a decrease in cytoplasm and numerous mitotic figures. × 260.

FIG. 13. Poorly differentiated epidermoid carcinoma of the spindle cell variety. × 260.

FIG. 14. Muco-epidermoid carcinoma showing nests of moderately well differentiated squamous cells with transition to mucus-containing cells. Several acinus-like structures may be seen. × 350.





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# MORPHOLOGIC AND FUNCTIONAL CHANGES ASSOCIATED WITH TRANSPLANTABLE ACTH-PRODUCING PITUITARY TUMORS OF MICE *

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Recently, Furth and associates[1] reported the occurrence of transplantable pituitary adenomas in mice exposed to ionizing radiation. This study concerns one of the two types of pituitary tumors induced by radiation which caused marked hypertrophy of the adrenal glands of the primary hosts.[2] The following data are on the morphology and function of such primary and transplanted tumors and the ultimate effects of tumors upon the hosts. It will be shown by a variety of endocrinologic techniques that these tumors synthesized adrenocorticotropic (ACTH) hormone. Prolonged uncontrolled secretory activity of the neoplasms resulted in hypertrophy and hyperfunction of the adrenal cortices of the hosts. The ultimate clinical manifestations of these changes were obesity, lymphopenia, cessation of estrual cycle, a diabetes insipidus-like syndrome, and a fatal, generalized, pyogenic infection. These features constitute a syndrome, characteristic in mice of hyperadrenocorticalism.

## MATERIALS AND METHODS

A total of approximately 300 $LAF_1$ male and female mice were used in this study. They were maintained in individual metabolic cages or in a colony and all received a standard diet consisting of Purina Chow and tap water ad libitum, supplemented weekly with fresh greens.

Details relating to the occurrence of the primary pituitary tumors have been reported by Upton and Furth.[3] The three primary hosts upon which this study is based were female $LAF_1$ mice which survived exposure to ionizing radiation from an experimental atomic detonation composed principally of gamma rays with a small admixture of neutrons, totaling 200 to 700 rep. All three mice were sacrificed within 24 to 30 months following exposure to radiation at a time when each appeared to be moribund.

In order to obtain additional data concerning the functional nature

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of the primary tumors, the bulk of the tumors was minced in saline solution and injected into the thigh muscles of male and female mice. The following descriptions are limited to the effects of the first 5 generations of transplants which produced the greatest degrees of adrenal hypertrophy and the most marked functional alterations. The tumors are still functional after 4 years during which time over ten successful passages were made.

All mice were necropsied. Since the major changes recorded were observed consistently, they will be described without stating the numbers of specimens observed except when the observations were based on small groups of animals. Most tissues saved for paraffin sections were fixed in Zenker's or Bouin's fluid. All staining procedures, unless specifically stated, are those described by Lillie.[4] Numerous animals have been preserved *in toto* for future studies.

## Results

### *Primary Tumors and Hosts*

Shortly following exposure to radiation, the three primary hosts showed moderate graying of the hair over the entire body. The mice were 24 to 36 months old when sacrificed. Grossly, each of the three pituitary glands measured about 5 mm. in average diameter. Histologically, the primary tumors, which henceforth shall be referred to as AtT* 1, 2, and 6, were similar and composed of uniform polyhedral cells. Mitotic figures were present. No specific chromophilic cytoplasmic granules could be identified by hematoxylin and eosin or by the Gude-Martins-Mallory stains.[5] Tumors 1 and 2 were confined to the sella turcica while tumor 6 extended into the floor of the sella and infiltrated into the tissues about the intracranial segment of the internal carotid artery.

The adrenal glands of the primary hosts were estimated to be twice normal size. Histologically, they showed marked hypertrophy of the fascicular and reticular zones. The cells of the zona glomerulosa were only slightly enlarged. The x-zone, usually present in the juxtamedullary portion of the adrenal cortex of female mice, was absent. The cells of the adrenal medulla were large and contained prominent chromaffin granules (Figs. 2, 4, 6, 8, 9, and 10).

### *Functional Effects of Transplanted Tumors*

Within 2 to 6 months following transplantation, at a time when the grafted tumors were barely palpable, a typical clinical syndrome de-

* Adrenotropic tumor.

veloped in both male and female mice. The changes consisted of obesity, polydipsia, polyuria, and lymphopenia. In female mice there was cessation of the estrual cycle. The magnitude of these changes during the 7-day period of maximum urine volume is recorded in Table I. Hyperglycemia and glycosuria occurred only in a few mice bearing transplants of the first generation of tumor strains 1 and 2. In all other tumor-bearing mice neither glycosuria nor hyperglycemia was observed. The results of studies of glucose tolerance, of sodium, potassium, and nitrogen balances, and of urinary steroid excretion are reported separately.[6-8]

### *Immediate Cause of Death*

Some mice died with no gross morphologic or histologic lesions, except hypertrophy of the adrenal cortex, to account for death. In these mice it was assumed that the electrolyte disturbances attending hyperadrenal cortical activity were fatal. Death in most tumor-bearing mice was preceded by a generalized pyogenic infection during which abscesses formed in the lungs (Fig. 13), kidneys, liver, myocardium (Fig. 11), endocardium (Fig. 12), pancreas, and adrenal glands. Microscopically, these lesions were composed of ne-

TABLE I

*Ranges of Functional Effects of Transplanted Tumors in Intact Mice*

| Tumor strain | No. mice | Food per day | | Water per day | | Urine per day | | Urine specific gravity | | Total leukocytes $\times 10^3$ | | Lymphocytes | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Min. | Max. | Min. | Max. | Min. | Max. | Min. | Max. | Min. | Max. | Min | Max. |
| | | gm. | gm. | ml. | ml. | ml. | ml. | | | | | % | % |
| Normal | 51 | 2.3 | 7.1 | 2.0 | 10.4 | 0.2 | 2.9 | 1,060 | 1,070 | 9.7 | 12.1 | 83 | 96 |
| 1 | 16 | 2.1 | 8.0 | 9.5 | 71.0 | 5.0 | 59.0 | 1,014 | 1,060 | 0.3 | 4.8 | 0 | 16 |
| 2 | 3 | 5.4 | 6.5 | 18.0 | 39.0 | 4.1 | 34.0 | 1,014 | 1,030 | 1.6 | 4.4 | 0 | 5 |
| 2 | 17 | 3.0 | 8.7 | 5.0 | 27.0 | 2.0 | 17.0 | | | | | 4 | 28 |
| 6 | 3 | | | | | 2.0 | 27.0 | | | 2.0 | 3.4 | 0 | 24 |

crotic amorphous débris and masses of Gram positive, periodic acid-Schiff (PAS) positive bacilli (Fig. 14). The leukocytic infiltration of tissues surrounding abscesses was not extensive and there was no evidence of delimiting fibroblastic activity. A diphtheroid organism (*Corynebacterium Kutcheri*) was repeatedly isolated from such lesions. Bilateral total adrenalectomy was the only effective means of controlling manifest infection. Slight infection could be controlled with terramycin in drinking water. This proved to be an excellent prophylactic measure.

*Morphology of Transplanted Tumors*

The transplanted tumors were small and at death usually weighed less than 500 mg. The tumors did not metastasize to regional lymph nodes or to other organs but infiltrated muscles at the site of implantation (Fig. 16). Histologically, the tumors were composed of relatively small cells which usually were arranged in cords about foci of necrosis. Necrosis was common and extensive in normal hosts, scant in adrenalectomized hosts. The nuclei were hyperchromatic and mitotic figures were present. The cytoplasm showed moderate diffuse basophilia when stained with azure-eosin mixture (pH 4.5), but contained no specific chromophilic granules when stained with the Gude-Martins-Mallory,[5] chromalum hematoxylin-phloxine, phosphotungstic acid-hematoxylin, periodic acid-Schiff, aldehyde fuchsin, and hematoxylin and eosin stains. By the usual criteria, the tumors can thus be considered to be chromophobic adenomas (Fig. 15).

Prolonged serial transplantation of tumor 2 (Table II) resulted in increased pleomorphism of neoplastic cells accompanied by marked increase in the maximum weight of the tumor and decrease in ACTH production as judged by adrenal weights and water intake of tumor-bearing mice.

*Morphologic Effects of Transplanted Tumors*

The weights of organs of intact mice bearing tumors are recorded in Table II. Most tumor-bearing mice were very obese. The maximum body weight of some mice reached more than 50 gm. as compared to a maximum of 35 gm. in control mice of the same age and sex. The body contour of tumor-bearing mice was rotund. There was a marked increase in the thickness of subcutaneous fat depots. The peritoneal fat was also increased as demonstrated by the observations that the mean weight of the testicular fat pad of 23 male tumor-bearing mice was 1.2 gm. as compared to a mean of 0.6 gm. in ten normal males of the same age.

The adrenal glands of all mice bearing transplanted tumors were

TABLE II

*Mean Organ Weights of Intact Mice Bearing Transplanted Tumors*

| Tumor strain | Sex | No. mice | Final body weight | Testicular fat body | Adrenal glands | Seminal vesicles | Testes | Uterus | Ovaries | Tumor |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| Normal | F | 13 | 23.4 | | 6.4 | | | 111 | 10.0 | 0 |
| | M | 10 | 34.8 | 0.6 | 4.6 | 325 | 212 | | | 0 |
| 1 (G 1–3) | F | 55 | 30.0 | | H | | | N | N | 200 |
| | M | 36 | 30.0 | | H | N | N | | | 200 |
| 2 (G 1–3) | F | 30 | 30.0 | | H | | | N | N | 200 |
| | M | 30 | 30.0 | | H | N | N | | | 200 |
| 2 (G 4–5) | F | 11 | 30.0 | | 16.6 | | | 86 | 5.6 | 1,300 |
| | M | 23 | 35.3 | 1.2 | 13.8 | 316 | 166 | | | 344 |
| 2 (G 7) | F | 22 | 35.1 | | 7.3 | | | N | N | 6,400 |
| 6 (G 1) | F | 20 | 30.0 | | H | | | | N | 200 |
| | M | 20 | 30.0 | | H | N | N | N | | 200 |

G = generation, H = hypertrophy, N = normal.

enlarged to approximately three times normal size. The cells of the zonae fasciculata and reticularis were greatly enlarged and almost completely depleted of lipid and stainable carbonyl groups as demonstrated by the direct Schiff and Ashbel-Seligman techniques. The cells of the zona glomerulosa were only slightly enlarged and, unlike the other zones, retained their lipid content (Figs. 3 and 19).

The ovary was moderately atrophic and contained only a few small and medium-sized follicles. Atretic follicles were plentiful. There were no large follicles or corpora lutea. Even the interstitial débris of old corpora lutea was lacking. The nuclei of the interstitial cells were pyknotic (Fig. 18). The uteri were normal grossly. Histologically, the tall columnar uterine epithelium often exhibited characteristic clear basal vacuoles. The associated endometrial stroma was usually edematous.

The vaginal mucosa never was completely cornified. The epithelium consisted of an inner zone composed of a few layers of cells of cuboidal basal type and an outer zone of mucus-containing cells (Fig. 17). Polymorphonuclear leukocytes could be identified in both epithelial zones and within the lumen of the vagina. In some female mice the breast tissue was hypertrophic. Both ductal and alveolar elements were at times enlarged and contained vacuolated eosinophilic secretions.

The testes, seminal vesicles, prostate, and epididymides were essentially normal histologically.

The structural changes in the kidneys were not as marked as the observed functional defects. There was no evidence of glomerular or vascular lesions of either nephrosclerosis or periarteritis nodosa. In mice with greatest urine volume there was moderate to marked dilatation of the distal convoluted and the collecting tubules. The kidneys of most mice which died spontaneously showed foci of acute pyelonephritis and abscesses related to the previously described terminal septicemia.

Histochemical staining procedures were carried out on renal tissue from a few tumor-bearing mice. The distribution and intensity of staining caused by acid and alkaline phosphatase, succinic dehydrogenase, and $\beta$-glucuronidase did not differ from controls. The normal pattern of PAS staining of the brush border of the proximal convoluted tubules was maintained. There was no evidence of androgen-induced changes in the epithelium of Bowman's capsule of female mice.

The skin revealed a consistent increase in the thickness of the subcutaneous panniculus. The hair cycle was in the inactive phase and no changes were found in the pigmentation. Since melanocytes were not readily found in most areas of skin, evaluation of cutaneous melanino-

genesis was difficult. One exception was in the corium of the perineum where large hypertrophic melanocytes were easily seen in both normal and tumor-bearing mice. Examination of the iris, choroid, and the pigmented layer of the retina revealed no consistent changes in tumor-bearing mice.

There was marked atrophy of the thymic cortex and the splenic follicles. The erythroid and myeloid components of the bone marrow were prominent and the spleen often contained prominent megakaryocytes (Fig. 20).

### *Synthesis of Pituitary Hormones by Tumors*

The biologic properties of the hormones synthesized by the tumors were first established by observations of the effects of the tumor upon the hypophysectomized host. Seventeen male and female tumor-bearing mice survived complete hypophysectomy for more than 3 weeks. During this interval there was no skeletal growth. At necropsy each animal showed atrophy of the gonads, seminal vesicles or uterus, and the thyroid gland, while the adrenal cortex was hypertrophied (Fig. 5; Table III). Histologic examination of

TABLE III

*Mean Organ Weights of Hypophysectomized Mice Bearing Transplanted Tumors*

| Tumor strain | No. | Sex | Body weight | Adrenal glands | Testes | Seminal vesicles | Ovaries | Uterus | Thyroid gland |
|---|---|---|---|---|---|---|---|---|---|
| | | | gm. | mg. | mg. | mg. | mg. | mg. | mg. |
| Control | 4 | F | 24.3 | 3.3 | | | 3.0 | 20.3 | A |
| Control | 1 | M | 18.0 | 2.4 | 19.8 | 24 | | | A |
| 1 | 1 | F | 18.4 | H | | | A | A | A |
| 2 | 4 | F | 17.5 | H | | | A | A | A |
| 2 | 5 | M | 25.0 | H | A | A | | | A |
| 2 | 1 | F | 22.0 | 19.0 | | | 3.0 | 25.0 | A |
| 2 | 2 | M | 23.5 | 16.7 | 92.0 | 44 | | | A |
| 6 | 2 | F | 20.0 | H | | | A | A | A |
| 6 | 2 | M | 25.0 | H | A | A | | | A |

H = hypertrophy, A = atrophy.

the tibial epiphyses revealed no cartilaginous thickening. This evidence indicates that the tumor synthesized and secreted ACTH but did not secrete gonadotropins, thyrotropin, or growth hormone.

Extracts of lyophilized tumor tissue were also assayed* for prolactin, gonadotropins, thyrotropin (TSH), adrenocorticotropin (ACTH), and melanocyte expanding hormone (MSH). There was no evidence of gonadotropins, prolactin, or TSH in 10 mg. of tumor powder. However, the concentration of ACTH and MSH activity in tumor extracts was about one tenth to one thousandth that of beef anterior lobe powder.

## Discussion

All the primary and transplanted tumors described in this paper are morphologically similar. They are composed of relatively smaller cells than the thyrotropin secreting tumors previously described.[9] The absence of specific intracellular granulation prohibits any conclusions concerning the cytologic origin of the tumor. The term chromophobe adenoma is obviously unsatisfactory for it merely indicates the absence of any positive histologic diagnostic criteria.

Although devoid of special cytologic features, these tumors can easily be characterized by their effects on intact and hypophysectomized hosts and by direct bioassay. They differ from previously reported tumors in that they contain no TSH but do possess ACTH and MSH activities, and when transplanted cause a distinctive syndrome in the host. The tumors resemble the normal pituitary body in that they can synthesize biologically active substances, but whether these hormones are identical with the compounds secreted by the normal pituitary gland remains to be demonstrated. It is apparent that the tumor cells were not under the absolute control of the neurohumoral mechanisms which constantly regulate the pituitary-adrenal axis[10] of the normal animal, since fatal adrenal cortical hyperfunction occurred in the tumor-bearing mice.

The rôle of the adrenal gland in the causation of the clinical syndrome has been illustrated previously in studies of adrenalectomized hosts.[11] Following the removal of the adrenal glands of these tumor-bearing mice, the number of circulating lymphocytes increased; the urine volume, which had been increased, returned to normal; periodic vaginal cornification reappeared, and the generalized infections were controlled. When mice were adrenalectomized prior to the onset of

* Assay for gonadotropins, TSH, and prolactin by Dr. R. W. Bates, National Institutes of Health; assay for ACTH by Dr. S. L. Steelman, Armour Laboratories.

polyuria, the lymphopenic-polyuric syndrome was not observed, nor did the mice become obese. These findings are supported by the recovery of increased amounts of corticosteroids and ketosteroids from the blood of tumor-bearing mice.[8]

Although the features of this clinical syndrome differ somewhat from the typical Cushing's syndrome of man, they correspond to known effects of adrenal corticosteroids. The diabetes insipidus-like state characterized by marked polydypsia and polyuria, unaccompanied by profound glycosuria, has been produced in dogs and rats by desoxycorticosterone and may indicate excessive mineralocorticoid activity.[12] Lymphopenia, thymic atrophy, susceptibility to infection, and the occasional occurrence of hyperglycemia in the first generation of tumors can most likely be attributed to glucocorticoid activity.[13,14]

The occurrence of ovarian atrophy and vaginal mucification seems to indicate inhibition of pituitary gonadotropins possibly by adrenal ketosteroids.[15] It should be noted that in hypophysectomized tumor-bearing mice there was atrophy of the seminal vesicles in spite of adrenal hypertrophy, which indicates that the adrenal ketosteroids exhibited little true androgenic activity.

The genesis of obesity in tumor-bearing mice as well as in Cushing's syndrome of man is obscure. Heilman and Kendall[16] reported the occurrence of obesity in certain strains of mice implanted with pellets of either 11-dehydrocorticosterone or corticosterone. Implantation of pellets of cortisone or cortisone plus testosterone did not result in any increase in total body fat. Since obesity was most prominent in groups of tumor-bearing mice showing the most marked adrenal cortical hypertrophy and was prevented by adrenalectomy, it is likely that the adrenal cortex played an important etiologic rôle. In a recent study, Mayer and associates[17] have shown that the extrahepatic fat was increased in mice bearing the same ACTH-secreting tumor as those reported in this paper and this was accompanied by decreased spontaneous activity of the mice. Moreover, fat was synthesized in such mice from labeled precursors even when the animals were starved. (It may be that some adrenal corticoids secreted by the stimulated adrenal glands are capable of causing obesity by direct metabolic effects.)

## Summary and Conclusions

Three primary pituitary adenomas of mice have been reported. Each tumor arose in the hypophysis of the primary host 24 to 36 months following exposure to total body radiation of 200 to 700 rep. The adrenal cortex of each mouse showed (1) hypertrophy of the zonae

fasciculata and reticularis, (2) little or no alteration in the zona glomerulosa, (3) atrophy of the x-zone.

Each primary tumor was grafted in series into the thighs of other mice of the same strain. In intact hosts the tumors caused a typical fatal syndrome characterized by adrenal hypertrophy, ovarian atrophy, thymic atrophy and lymphopenia, obesity, polydipsia and polyuria, cessation of estrual cycle, and susceptibility to infections. In hypopysectomized hosts, the transplanted tumors were shown to secrete ACTH.

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[ *Illustrations follow* ]

## LEGENDS FOR FIGURES

FIG. 1. Adrenal gland of normal adult $LAF_1$ female mouse. Hematoxylin and eosin stain. × 105.

FIG. 2. Hypertrophic adrenal gland of the primary host which was the source of adrenocorticotropic tumor 1 (AtT-1). Hematoxylin and eosin stain. × 105.

FIG. 3. Hypertrophic adrenal gland of mouse bearing transplanted adrenocorticotropic tumor 1 (AtT-1). Hematoxylin and eosin stain. × 105.

FIG. 4. Hypertrophic adrenal gland of the primary host which was the source of adrenocorticotropic tumor 2 (AtT-2). Hematoxylin and eosin stain. × 105.

FIG. 5. Hypertrophic adrenal gland of hypophysectomized (60 days) mouse bearing transplanted adrenocorticotropic tumor 1 (AtT-1). The architecture of the adrenal cortex is maintained despite removal of the anterior pituitary gland. Hematoxylin and eosin stain. × 105.

FIG. 6. Hypertrophic adrenal gland of the primary host which was the source of adrenocorticotropic tumor 6 (AtT-6). Hematoxylin and eosin stain. × 105.

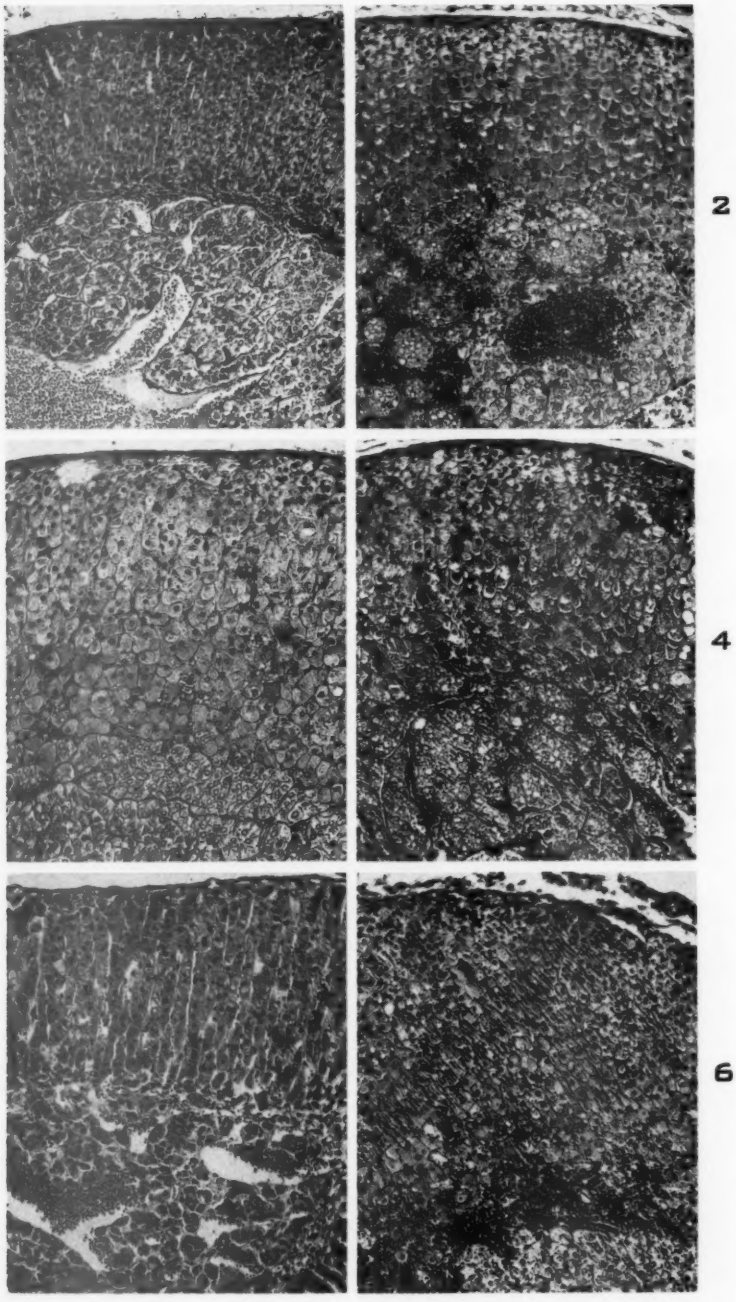
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FIG. 7. Zonae glomerulosa and fasciculata of normal adult female $LAF_1$ mouse. Hematoxylin and eosin stain. × 500.

FIG. 8. Zonae glomerulosa and fasciculata of the primary host which was the source of AtT-1. Hypertrophy confined chiefly to the zona fasciculata. Hematoxylin and eosin stain. × 500.

FIG. 9. Zonae glomerulosa and fasciculata of the primary host which was the source of AtT-2. For comparison with Figures 7 and 8. Hematoxylin and eosin stain. × 500.

FIG. 10. Zonae glomerulosa and fasciculata of the primary host which was the source of AtT-6. For comparison with Figures 7 and 8. Hematoxylin and eosin stain. × 500.

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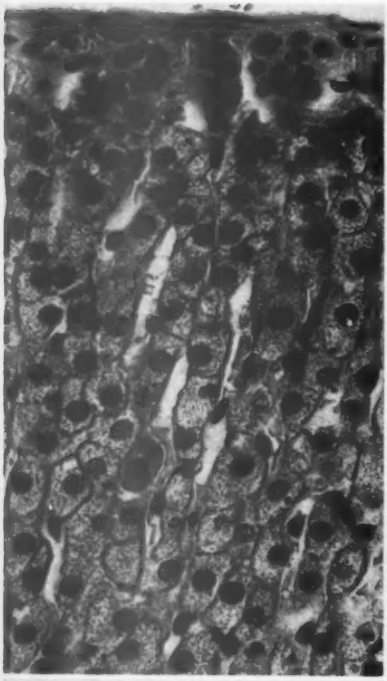

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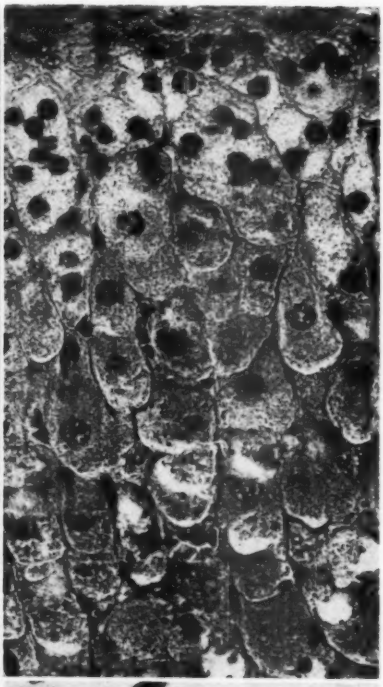

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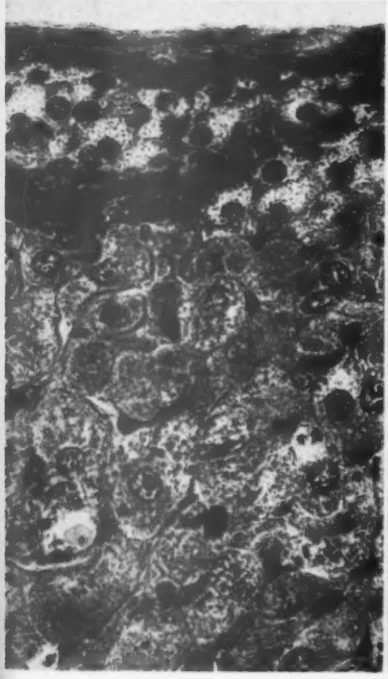

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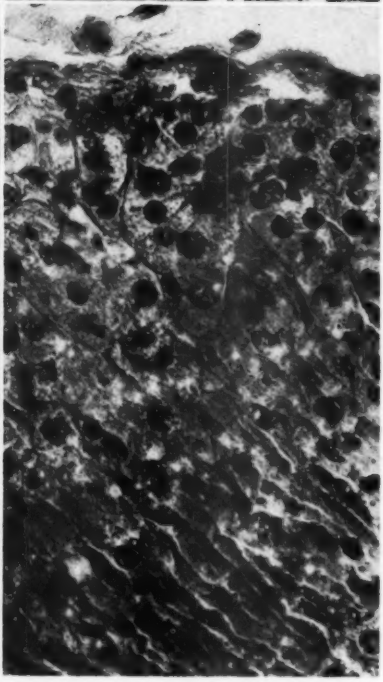

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FIG. 11. Myocardial abscess in heart of mouse bearing transplanted AtT-1. Gram's stain. × 100.

FIG. 12. Acute mitral bacterial endocarditis in heart of mouse bearing transplanted AtT-6. Hematoxylin and eosin stain. × 105.

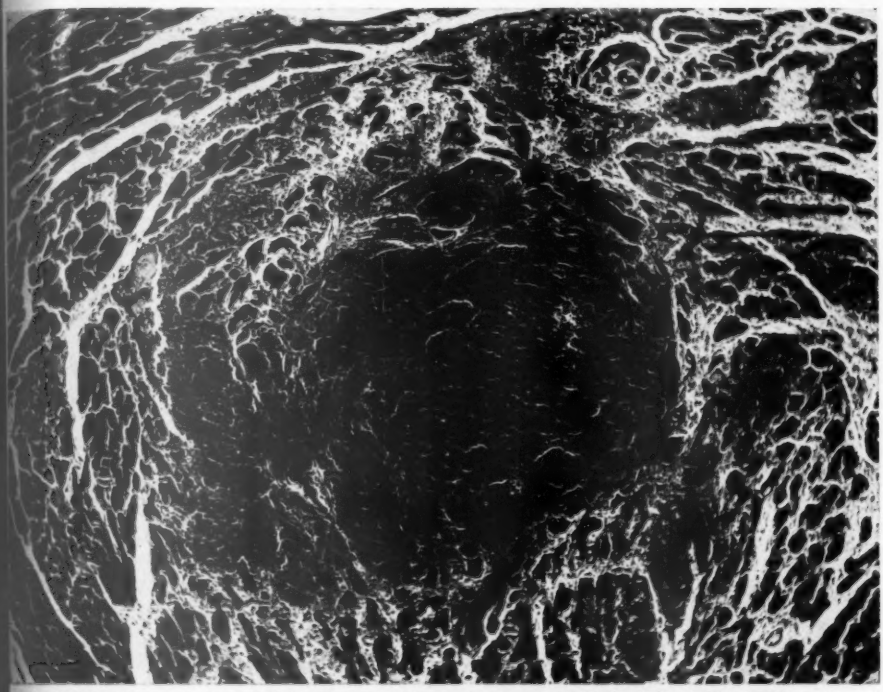

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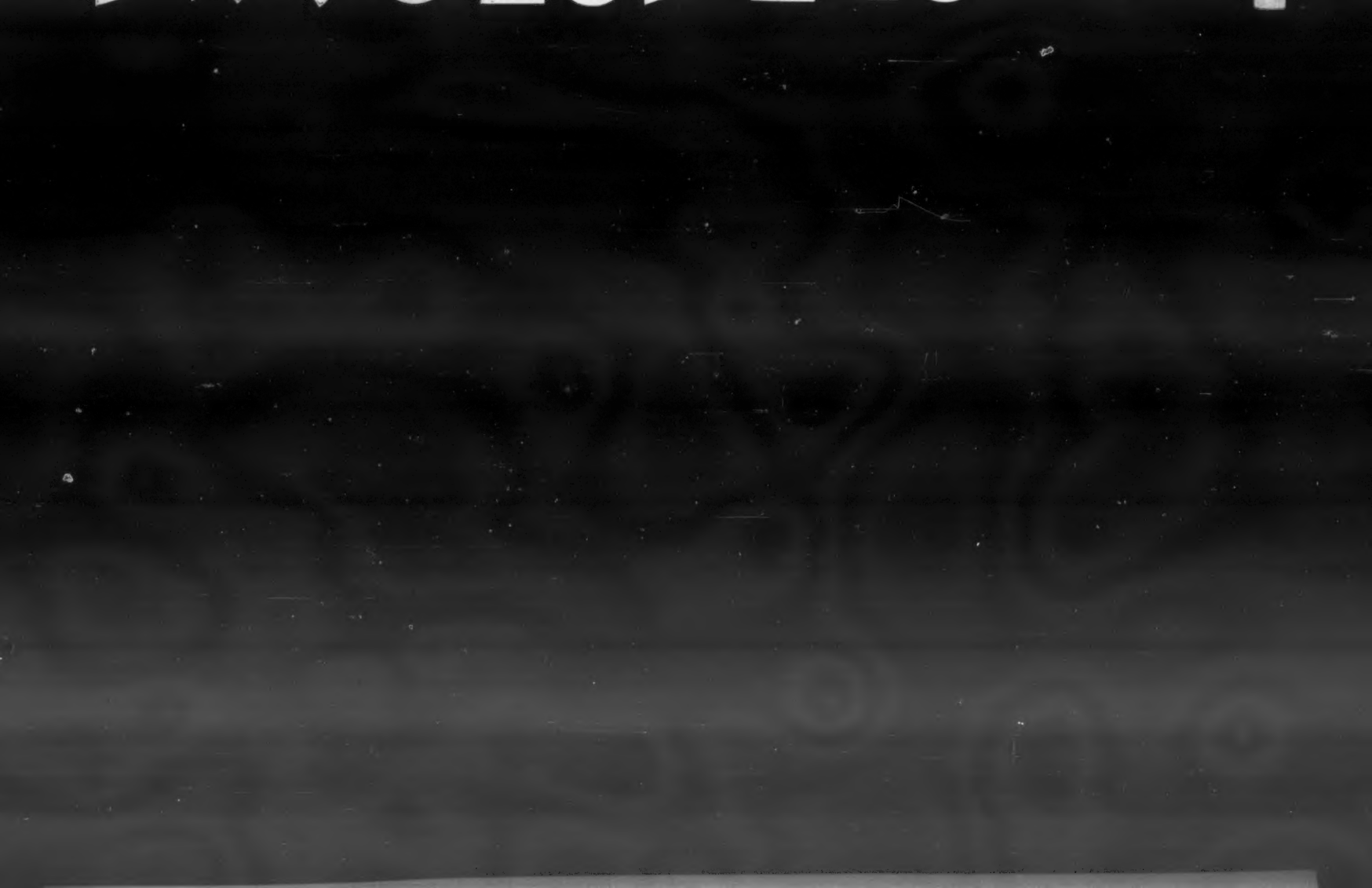
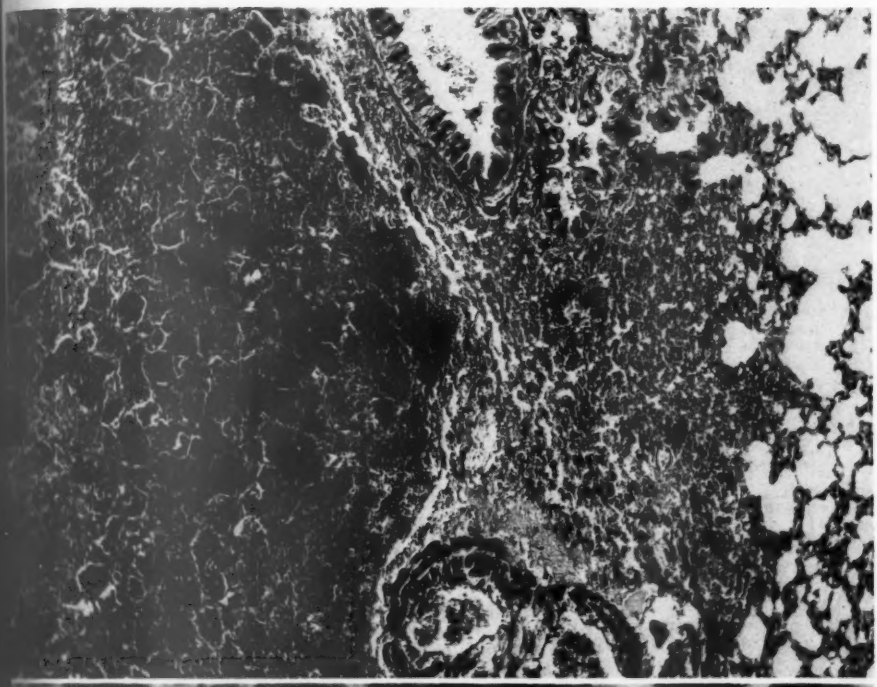
FIG. 13. Pneumonia and pulmonary abscess in lung of mouse bearing transplanted AtT-1. Gram's stain. × 105.

FIG. 14. Gram-positive bacilli in pulmonary abscess seen in Figure 13. Gram's stain. × 1,700.





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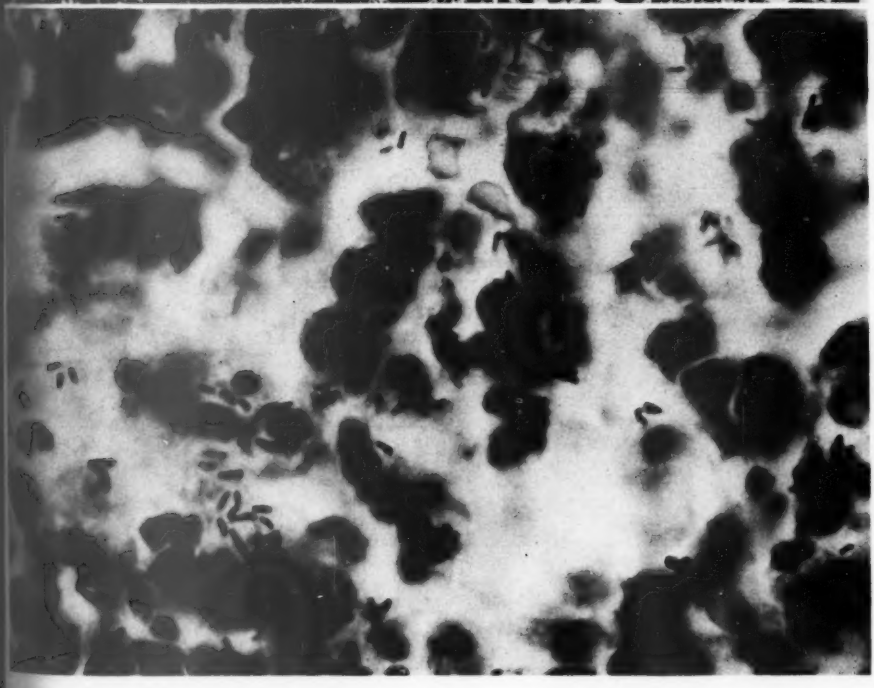

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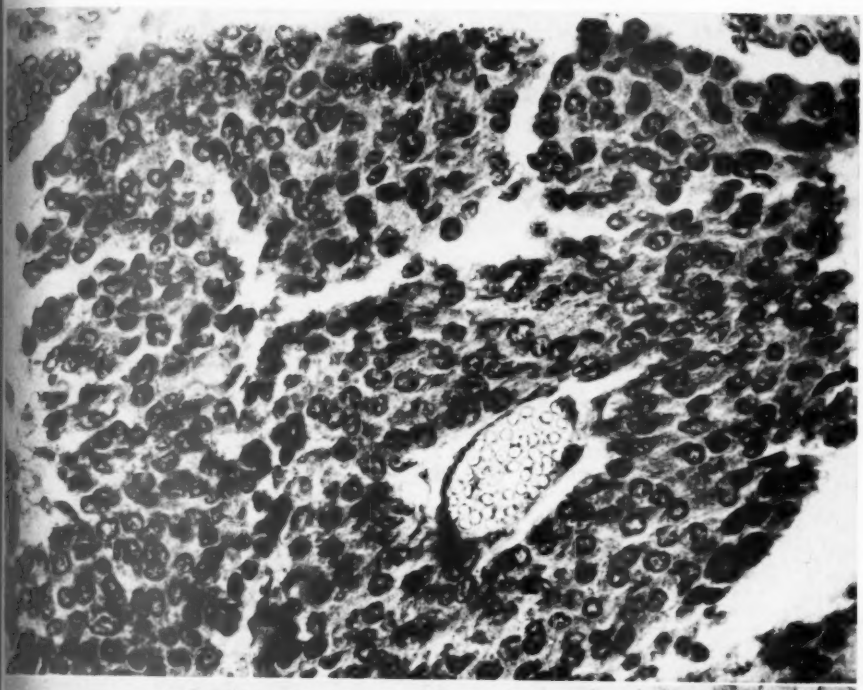
FIG. 15. Transplanted adrenocorticotropic tumor 2 (AtT-2) dissected from thigh of adult $LAF_1$ mouse. The nuclei are large, vesicular, and moderately pleomorphic. No specific chromophilic granules can be identified in the cytoplasm. The stroma is sparse. Periodic acid-Schiff and hematoxylin stains. × 550.

FIG. 16. Invasion of thigh muscles by transplanted adrenocorticotropic tumor 2 (AtT-2). For comparison with Figure 15. Periodic acid-Schiff and hematoxylin stains. × 105.



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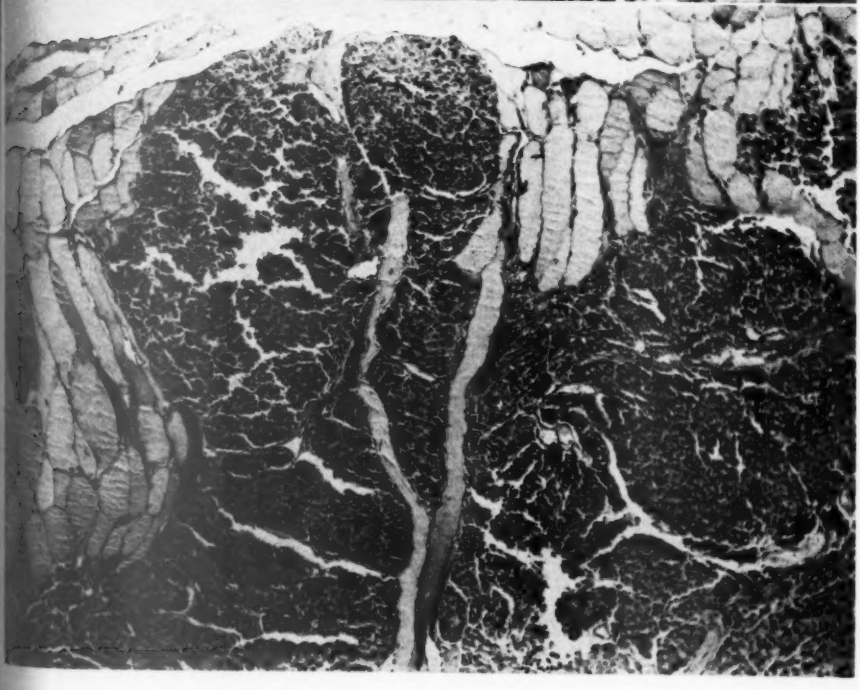

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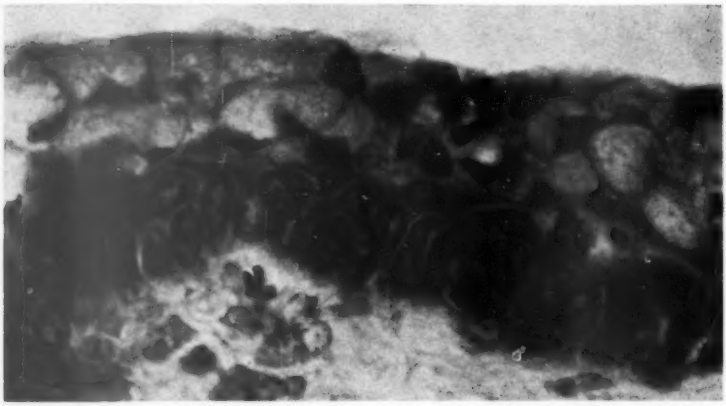
FIG. 17. Vagina of mouse bearing transplanted AtT-2. Mucin ladened cells compose the superficial layers of the epithelium. Hematoxylin and eosin stain. × 1,580.

FIG. 18. Ovary of mouse bearing transplanted AtT-2. There is atrophy of the interstitial tissue and atresia of follicles. No cystic follicles or corpora lutea are present. Hematoxylin and eosin stain. × 73.

FIG. 19. Adrenal cortex of mouse bearing transplanted AtT-2. The zonae fasciculata and reticularis are hypertrophic and depleted of lipids. Lipids remain in outer rim of zona glomerulosa. Frozen section and oil red O stain. × 105.

FIG. 20. Spleen of mouse bearing transplanted AtT-2. Megakaryocytes are present. Lymphoid follicles are atrophic. Hematoxylin and eosin stain. × 100.



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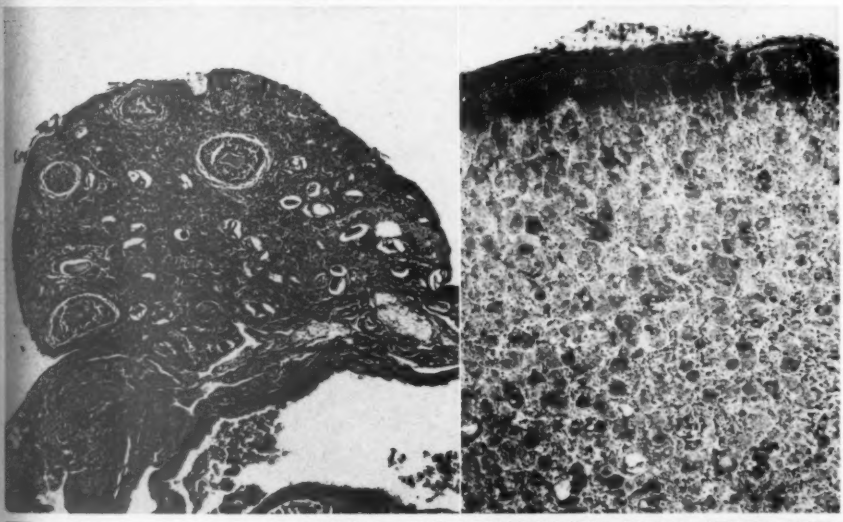

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# EXPERIMENTAL ANEMIC INFARCTION OF THE LUNG

## The Morphologic Evolution of True Infarcts of the Lung Produced with a Halogenated Hydrocarbon *

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A formidable list of experiments beginning with the studies of Virchow[1] in 1847 attest to the difficulty of consistently inducing infarction of the lung following simple occlusion of the pulmonary artery. Failure of the lung to undergo infarction has been noted after experimental embolization with a large number of different substances.[2-19] Only the report of Steinberg and Mundy[20] suggests that if a sufficient number of emboli are introduced, infarction may result. Their use of lead shot as the embolic material may be open to criticism because the weight of the shot alone might have contributed to the infarction by altering pulmonary hemodynamics. It also has been shown that ligation of the pulmonary artery does not cause infarction.[17,21,22]

The early comprehensive studies of Karsner and Ash,[14] and the subsequent work of others,[16,23] have shown that pulmonary necrosis does not occur when the arterial circulation is blocked unless circulatory stasis is also present in the involved pulmonary segment. Karsner and Ash found it possible to cause infarction in dogs with occluded pulmonary arteries by ligating the pulmonary veins or by compressing the pulmonary tissue by means of an artificial pleural effusion. Interference with the blood flow from the bronchial arteries alone did not cause pulmonary infarction. The latter observation was confirmed by Ellis *et al.*,[17] who further concluded that an impaired bronchial arterial circulation might actually be a contributing factor in the development of pulmonary infarction after pulmonary embolism. Moses[16] was able to produce infarction by external compression of the thorax and embolization of the pulmonary arterioles in rabbits. This interference with the hemodynamics of the lung resulted in various degrees of infarction. Of necessity, experimental operative procedures on the pulmonary vasculature are confined to the large vessels, and compression of the thorax adds a variable which is hard to control. Both measures produce more extensive alterations in pulmonary circulation than could be expected with a clinically significant infarct in man.

Attempts at producing pulmonary infarcts by chemical means are

* Received for publication, February 1, 1957.
† Now at the National Cancer Institute, Laboratory of Pathology, Bethesda, Md.

not new. Karsner and Ash[14] mentioned that in 1887, Orth,[24] utilizing paraffin emboli, "mixed various chemical irritants with the emboli and obtained fairly constant results." Approaching the problem with an experimental model more like the clinical situation of passive hyperemia with superimposed blood clot emboli, Chapman *et al.*[23] were able to produce infarcts in the lungs of three of eight dogs by administering large doses of alphanaphthylthiourea (ANTU) just prior to the release of intravascular blood clots into the peripheral venous circulation. Unfortunately, the level of ANTU necessary to produce sufficient pulmonary hyperemia and edema caused death of the animals in 72 hours or less, hence the development of the lesions could not be followed.

During a course of investigations on the toxicity of halogenated hydrocarbons it became evident that certain of these compounds were capable of producing multiple anemic pulmonary infarcts in rabbits and dogs after single, sublethal, intravenous doses. This paper describes the progress of vascular and parenchymatous alterations produced in the lungs of rabbits by the halogenated hydrocarbon, hexachlorotetrafluorobutane. The fact that infarcts invariably occur, and that the extent of the lesions can be controlled by the dose of the hydrocarbon, makes this phenomenon a ready quantitative means of studying pulmonary infarction.

## Materials and Methods

Sixty-two healthy New Zealand white rabbits were used as the experimental animals. The rabbits were of both sexes, weighed 1,600 to 2,400 gm., and were maintained on unrestricted diets of commercially pelleted rabbit-food and water.

The animals were sacrificed by intraperitoneal injections of nembutal after the experimental procedure. The lungs, heart, liver, and kidneys of all rabbits and the other viscera of most animals were examined histologically. All tissues were fixed in 10 per cent neutral formalin, and the lungs were filled with fixative through the trachea. Sections of the lungs were made from the periphery to the hilus through the greatest diameter of each lobe or of the infarct. Paraffin embedded blocks were sectioned at 5 to 7 $\mu$ and stained with hematoxylin and eosin. Selected sections were prepared with the Weigert elastic tissue method, the Masson trichrome stain, or alizarin red; other tissues were sectioned after freezing and were stained for fat with oil red O.

Twenty-five rabbits that had been maintained in a like manner were sacrificed and examined during the course of the study for controls. The lungs of the control rabbits showed no primary vascular lesions, but occasionally small collections of lymphocytes were seen around large blood vessels. In rare cases healed granulomas were present in

the lungs. Coccidial granulomas were present in the livers of about 15 per cent of the animals, and a small group showed lesions of the brain and kidney in which *Encephalitizoon cuniculi* could be identified. Such incidental lesions showed scarring of distinctive character and sufficient duration to cause little confusion with lesions primarily due to the chemical agent. In the experimental animals no other lesions or clinical signs were noted which could not be related to the pulmonary infarcts.

Hexachlorotetrafluorobutane is an oily liquid at room and body temperatures with a density of 1.828 at 25° C. The compound is immiscible in water and miscible in the common organic solvents. It is relatively inactive chemically when compared with other halogenated hydrocarbons. When the hydrocarbon is agitated in blood it forms a coarse emulsion which remains dispersed for some time. Presumably such an emulsion is formed when the compound passes through the right chambers of the heart. The hydrocarbon has no measurable effect on the clotting time of whole blood. In all studies the hydrocarbon, hexachlorotetrafluorobutane, was administered as a single injection into the marginal ear vein. Dosages ranged from 0.01 to 0.40 ml. per kg.

## Quantitative Studies of the Pulmonary Lesion

Table I charts gross evaluations of the lesions noted in rabbits sacrificed at various intervals following the injection of single graded doses of the compound. Discrete areas of hyperemia, which were surrounded by grossly unaffected lung tissue, could be detected within 12 hours. These well demarcated areas, the precursors of ischemic infarcts, were distributed indiscriminately throughout all lobes of the lungs, but within the lobes they were commonly localized in the acute angles formed by the sharply reflected pleural surfaces (Fig. 1). This peripheral location caused the lesions to be pyramidal with apices formed by the pleural margins and convex bases directed toward the hilus of the lobe. Within 24 hours true infarcts had developed with characteristic wide based ischemic lesions separated from unaffected lung tissue by prominent borders of hemorrhagic consolidation. Because the lesions were discrete and distinguishable from the adjacent unaffected lung, their extent could be roughly measured. The infarcts varied in size and number with the dose. The usual infarct, resulting from a dose of 0.10 ml. per kg., measured from 0.5 to 1.0 cm. in its greatest dimension. Table I indicates that the total surface area of infarction estimated as a percentage of the surface of both lungs, was related to the amount of the hydrocarbon administered. When 0.40 ml. per kg. was given, the lesions were too extensive to be compatible with life and

TABLE I

*Gross Evaluation of the Lung Surface Involved Following Single Graded Doses of Hexachlorotetrafluorobutane*

| Dose ml./kg. | Time after injection | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2–6 hrs. | 7–12 hrs. | 1 day | 2 days | 3 days | 4 days | 6–7 days | 1–2 mos. | 3 mos. | 4 mos. |
| 0.01 to 0.02 | | | | | o<br>+<br>+<br>+× | +<br>+ | | | | |
| 0.05 | | | | o<br>+×<br>+× | ++<br>+×<br>+× | +<br>+×<br>+×<br>++× | ++<br>+××<br>+×× | | | |
| 0.10 | | o<br>+<br>+<br>+ | +<br>++<br>+× | +× | +××<br>+×× | +××<br>+××<br>+×× | +×★<br>+×★<br>+×★ | ★<br>★ | | ★ |
| 0.20 | +<br>+<br>++<br>++ | ++<br>++ | +××<br>+××× | | +××<br>+××<br>++× | | +×★<br>+×★ | ★<br>★★ | ★★<br>★★<br>★★★ | |
| 0.40 | | ++++ | +××× | +×××<br>+×××<br>+××× | +××× | | | | | |

\+ = Hemorrhagic (incomplete) infarction.
× = Hemorrhagic and anemic infarction.
★ = Parenchymatous scarring.

\+ = Less than 10% lung surface involved.
++ = Less than 20% lung surface involved.
+++ = Less than 30% lung surface involved.
++++ = More than 30% lung surface involved.

three of the six rabbits died within 48 hours. When doses of less than 0.10 ml. per kg. were given, the compound did not consistently cause grossly detectable infarcts. It was evident that infarcts, quantitatively comparable to those seen in man, could be produced with intravenous injections of 0.05 to 0.10 ml. per kg. of the hydrocarbon.

For descriptive purposes, infarcts caused by doses of 0.20 ml. per kg. were also studied to follow the progress of the lesion. A total of 42 rabbits subjected to the dosage range of 0.05 to 0.20 ml. per kg. were sacrificed at intervals of 2, 6, 8, 12, 18, and 24 hours, and at 24-hour intervals for the first week after injection. Subsequently, four rabbits were sacrificed during the first and second month, three at the end of the third month, and one, 4 months after the single injection. Although the extent of the involved areas varied with the dose, the sequence of morphologic alteration was similar.

## Morphologic Evolution of the Lesion

### *Early Development*

During the first 6 hours a patchy hyperemia of the lung parenchyma was the only gross evidence of pulmonary involvement. Histologic analysis demonstrated this change as an intense stuffing of the alveolar capillaries with erythrocytes; the alveolar lumina remained free of fluid (Fig. 3). At the periphery of the hyperemic areas minute arterioles also were filled with cells, but here the blood vessels were packed with leukocytes and swelling of the lining endothelial cells could be seen in vessels measuring 100 $\mu$ in diameter.

Within 12 to 24 hours the mottled hyperemic areas merged to form semi-translucent areas of ischemic necrosis, demarcated from grossly normal lung by wide red bands of hemorrhagic tissue. A similar but narrower band of congestion and hemorrhage, derived from the small pleural vessels, enabled a thin sheet of subpleural pulmonary tissue to survive the first 24 hours. The areas of infarction maintained a semblance of normal architecture; the capillaries were filled with masses of barely distinguishable conglutinated erythrocytes; the cells of the alveolar wall possessed pyknotic nuclei, and the poor staining character of coagulative necrosis was evident (Fig. 2). Within the necrotic areas the alveolar lumina became filled with an eosinophilic serous exudate almost devoid of formed cellular elements. A sharp margin of nuclear detritus separated the areas of coagulative necrosis from surrounding zones of hyperemia and hemorrhage. In the wide periphery of surviving hemorrhagic tissue there was an extension and intensification of the vascular endothelial changes noted in the first 6 hours (Fig. 5). Damage to the endothelium of veins draining the areas of infarction as well

as of the arteries was evident. Outside the areas of ischemic necrosis both arteries and veins, with diameters of 200 $\mu$ or less, were lined by swollen elevated endothelial cells infiltrated with polymorphonuclear leukocytes which had migrated through the arterial walls. Internal elastic membranes appeared intact and the muscular walls were not necrotic. The dilated lumina were filled with conglutinated red cells, but true thrombi were not evident in either arteries or veins at this early stage. The oil red O stain did not show lipid in the vascular lumina.

At 48 to 96 hours the thin border of nuclear fragments and leukocytic infiltration, which separated the region of necrosis from the hemorrhagic zone, was invaded by granulation tissue. By the third day, additional changes had occurred in the surrounding pulmonary vessels. Cellular infiltrations, with neutrophilic leukocytes predominating, extended through the vascular walls and into the perivascular spaces. Marked edema involving adventitia and perivascular lymphatics added to the pattern of vascular alteration (Fig. 6). Endothelial damage extended well beyond the zone of hemorrhage, and fibrin thrombi formed plaques over the partially destroyed endothelial linings of those arteries and veins with diameters as great as 300 $\mu$. Similar thrombi occluded vessels up to 200 $\mu$ in diameter. Only after the establishment of the fully developed infarct, did vessels supplying these areas contain formed occlusive thrombi. The bronchial arteries remained free of concomitant lesions.

Progressing from the third day through the first week, septal cells of the intact alveoli, just beyond the border of granulation tissue, showed an intense hyperplasia. The alveoli were then lined by cuboidal cells with large vesicular nuclei and basophilic cytoplasm. The altered septal cells were frequently multinucleated but only rare mitotic figures could be found. Where alveolar ducts were involved, the "adenomatosis" was associated with condensations of alveolar exudate which lined the ductal walls simulating hyaline (eosinophilic) membranes; these were most prominent in the lungs of rabbits which received doses of 0.40 ml. per kg. (Fig. 8). A profuse interstitial inflammatory response was invariably associated with the alveolar hyperplasia. The affected alveolar walls were infiltrated with neutrophilic and mononuclear leukocytes, but the lumina contained only serous exudate and desquamated septal macrophages. The transition from a normal to a hyperplastic state of the walls was relatively uniform along the margin of the infarct, but where respiratory bronchioles were involved the epithelial proliferation was most prominent and proliferating epithelium extended toward the hilus. Distorted terminal

bronchioles exhibited hyperchromatic columnar cells, some of which contained mitotic figures. There were also stratification of the epithelium and squamous metaplasia. The bronchiolar lumina frequently contained cellular detritus. The necrosis of infarction did not extend sufficiently to involve bronchi with cartilage in their walls.

By the end of the first week, replacement of the necrotic pulmonary tissue by connective tissue was well underway, but the marginal zones of septal hyperplasia and the peripheral areas of incomplete hemorrhagic infarction persisted. Many of the thrombosed vessels became canalized and bridged with fibroblasts (Fig. 7), and only a residuum of inflammatory reaction was evident at the margin of the shrinking scars and in the surrounding bronchioles.

### *End Result*

Lesions varied only in intensity in the nine animals sacrificed from the 20th day to the 4th month. Grossly, they were marked by contracted opaque white scars at the periphery of the lungs with stellate fissures following vascular distribution into the deeper pulmonary tissue. The scarred areas contained loose collagen in which many hemosiderin-laden macrophages had been trapped, and cystic spaces with poorly defined linings were found. Elastic tissue was abundant and showed the irregular pattern of condensation described in cicatrized infarcts of the human lung[25] (Fig. 9). The end result in the involved pulmonary arteries was a prosaic *endarteritis obliterans*.

Arteries and veins, with diameters of 300 $\mu$ and less, were found leading to the scars in which the lumina had been largely obliterated by fibrous tissue (Fig. 4). Small recanalized channels were trapped in the cicatrices and exhibited bizarre patterns of reduplicated and disoriented elastic tissue. In larger vessels organized thrombi formed eccentric plaques, but these showed no evidence of progression. Only vessels which were obviously related to the scarred infarcts showed intrinsic fibrosis. In addition to these features of healed infarcts, two striking changes were noted consistently, namely, persistent epithelial hyperplasia and focal ossification in the scars.

At the inner margins of the fibrous scars, proliferative activity of both the alveolar walls and bronchioles was more evident than during early phases of repair. Although some of this apparent increase in activity could be due to contracture of the scar, mitotic figures could still be found and a wider zone of hyperplastic alveoli separated the cicatrix from the unaffected lung tissue (Figs. 4 and 12). In the air spaces partially trapped by marginal sclerosis, the hyperplastic foci were formed of compressed respiratory channels lined with ciliated

columnar epithelium or hyperchromatic cuboidal epithelium; the two forms were frequently contiguous. Atypical squamous metaplasia also suggested protracted epithelial activity (Fig. 11). There was insufficient evidence of unrestrained growth to consider the lesions malignant and there was no indication of anaplasia or invasion. The bronchiolar growth was reminiscent of the proliferative activity of biliary epithelium commonly seen in progressive hepatic scarring. Alveolar fetalization was noted also in pulmonary parenchyma separated from the scars by normal lung tissue (Fig. 10). These isolated foci of septal hyperplasia were invariably associated with the smallest bronchioles proceeding to or from areas of infarction. Usually the change consisted of a simple conversion of the alveolar lining to a single layer of cuboidal epithelium unaccompanied by inflammatory reaction.

Calcification was first noted in the healing infarcts of rabbits sacrificed on the seventh day of the experiment. In the surviving air spaces in the zone of the granulation tissue, the trapped cellular exudate was partially broken down to a granular basophilic débris. The alizarin red stain indicated a sufficiently large amount of calcium in some of these areas to color the amorphous material red. Still later, during the first month, calcareous deposits could be noted in tags of granulation tissue which protruded into dilated, and presumably occluded, bronchiolar passages. Fragments of calcium-ladened material had also been carried to adjacent lymphatics and a secondary granulomatous response to crystals stainable with alizarin red could be seen in local lymphoid nodules (Fig. 13). In all sections of the scars in the lungs of the seven rabbits sacrificed during the second, third, and fourth month, gradations from calcareous deposits to ossified fragments could be found. When ossification was present the particles of bone were usually surrounded by scar tissue, but in rare instances one margin would exhibit a layer of cuboidal epithelial cells and disorganized calcium, indicating a transition of intraluminal calcareous deposit to bone (Fig. 14). Not only was there a temporal evolution in the development of ossified nodules, but transitions were apparent in the various levels of a scar, ranging from the calcium-ladened intraluminal tags of granulation tissue of the viable deep tissues to ossified particles imbedded in the collagenous tissue of the periphery. The bony fragments contained well formed osteocytes but no distinct haversian systems. They were generally asymmetric but roughly ovoid and measured from 90 to 450 $\mu$ in diameter. These measurements corresponded in size to the previously noted intraluminal deposits of calcium.

## Auxiliary Experiments

To ascertain that the anemic infarcts were not the result of peculiar anatomical characteristics of the rabbit lung, two mongrel dogs were given intravenous injections of the hydrocarbon at doses of 0.20 ml. per kg. The dogs were sacrificed after 4 days. Gross and microscopic examination of the lungs of both dogs showed anemic infarcts similar to those found in the treated rabbits. The lesions differed only in that wider zones of hemorrhage circumscribed the areas of necrosis, and the infarcts were not confined to the wedge-shaped areas of pulmonary tissue. In these respects the lesions resembled more closely the infarcts of man.

Screening experiments indicated that other halogenated hydrocarbons were capable of causing infarcts in the lungs of rabbits. Tetrachloroethane and tetrachlorodifluoroethane,* at equivalent doses, caused lesions which were similar to those of hexachlorotetrafluorobutane. Carbon tetrachloride also caused local necrosis of the lung in intravenous doses of 0.10 ml. per kg., but the process here was accompanied by a diffuse cellular inflammatory response. Moreover, the vascular distribution of the lesion and the well demarcated anemic infarcts were absent.

## Discussion

It seems unlikely that simple occlusion of the larger vessels was responsible for the experimental infarcts. This conclusion is supported by the fact that infarcts developed prior to the formation of detectable thrombi and before visible damage to the endothelium of muscular vessels became evident. The many prior investigations on embolism[1-24] also suggest this probability. Two other explanations should be considered. Increased coagulation or sludging of the blood might have been responsible for complete occlusion of the vessels at the capillary level. This seems unlikely since our *in vitro* studies on whole blood showed that even at 1:10 concentrations of hydrocarbon in blood there was no decrease in clotting time or evidence of red blood cell agglutination. Direct chemical injury to the capillaries, and perhaps to the entire septal wall, seems a more feasible explanation. Presumably minute immiscible globules of the hydrocarbon were carried to the involved vascular segments and there were spread sufficiently thin in the slowing capillary stream to allow direct action on the capillary endothelium. The paucity of inflammatory response or hemorrhage in the necrotic

* Kindly supplied by the "Kinetic" Chemical Division of E. I. DuPont de Nemours & Co., Wilmington, Delaware.

areas, coupled with the vascular distribution of these areas of dead tissue, indicates sudden complete necrobiosis of tissue from total cessation of the circulation. The subsequent inflammatory reaction in the arterioles and venules outside the area of necrosis supports this thesis of direct chemical injury to the vessel wall.

It should be emphasized that the inflammatory process in the vessels was an acute endarteritis and the end result was a simple obliterative fibrosis. The early vascular lesions could be considered a true necrotizing panarteritis but the final process was not a progressive sclerosis.

The fact that hyaline membranes were found in the terminal bronchioles bordering infarcts indicates that this substance can be a local intrinsic product rather than aspirated material. It is conceivably attributed to anoxia which Tregillus[26] and Arey and Dent[27] suggested as a cause of hyaline membrane disease in the newborn infant.

The persistent hyperplastic response of the pulmonary wall in the region of the healed infarcts is not unique. A similar reaction to injury has been noted in the lung with bronchiectasis, infectious granuloma, and atelectasis, as well as infarcts.[28] Whether this response is an abortive attempt at regeneration is a moot point, but we are impressed by the fact that mitotic activity continues in these hyperplastic cells for as long as 4 months after the original injury. King[29] believes that cicatricial fixation of pulmonary tissue is the principal predisposing factor to bronchiolar hyperplasia, but in our studies hyperplasia was an early reaction which occurred long before a fixing scar was formed. Raeburn and Spencer[28] and Zatuchni *et al.*[30] consider foci of bronchiolar hyperplasia as probable sites for the development of bronchiolar carcinoma. Certainly similar hyperplastic responses to inflammation have been linked to the onset of malignancy in pseudo-epitheliomatous hyperplasia of the skin which follows arsenical and irradiation injury, and in regenerative activity of the liver in hepatic cirrhosis. It is hoped that further studies on experimental chemical infarction will offer a means of testing this hypothesis.

The chronologic sequence in the development of the ossified fragments from calcified intraluminal exudate establishes the origin of these interesting formations, but it is difficult to form conclusions concerning the mechanism of their development. Dystrophic calcification and ossification of devitalized tissue is common in man and other animals. Surely tissue anoxia must play a rôle, but further speculation along chemical lines would be presumptuous in view of our present ignorance of bone formation.[31] The calcific lesions differ from those of *mikrolithiasis alveolaris pulmonum*[32] in that the characteristic laminated organic matrix is absent, although their formation from intra-alveolar

exudate might be the same. Structurally, the bony lesions resemble the osseous deposits in the lungs of cases with mitral stenosis, a provocative suggestion in the light of the coincident capillary thrombi, eosinophilic membranes, and organized intra-alveolar exudate found in lungs of individuals with the cardiac lesion.[33] It is interesting to note, too, a resemblance to the incidental bone fragments in the lungs of laboratory animals described by Innes *et al.*[34] These workers observed that the lungs of rabbits (animals from the same colony as those used in our experiments) were remarkably free of bony fragments (1 in 126 rabbits) as compared to the other species studied. Such incidental deposits of bone are distinguished by their lack of inflammatory residue.

Although the anemic infarcts produced by hexachlorotetrafluorobutane are oddities both in incidence and pathogenesis, in every way the lesion parallels the classical progress of an infarct in an organ nourished by end arteries. Unlike septic embolism, the parenchymatous inflammatory response was obviously a reaction to dead tissue rather than to the original injury. The phenomenon offers a convenient controllable method for producing blocked vascular segments of lung tissue, comparable to the typical pulmonary infarct of man and without the disadvantages of opening the thorax surgically and interrupting major pulmonary vessels. It also proves that true anemic infarcts without superimposed hemorrhage can occur in the lung.

## Summary

Anemic infarcts were produced in the lungs of rabbits and dogs by a single sublethal intravenous injection of hexachlorotetrafluorobutane. The evolution of the lesion is the same as that seen in the classical anemic infarction of organs possessing an end-arterial structure. The fact that infarction occurred prior to development of thrombi suggests that the lesion is the result of direct chemical action on the capillary bed rather than the result of simple thrombosis. Persistent hyperplasia of the adjacent pulmonary septa and ossification of dystrophic calcium deposits are features of the healed infarct which enhance the value of this phenomenon as an experimental tool.

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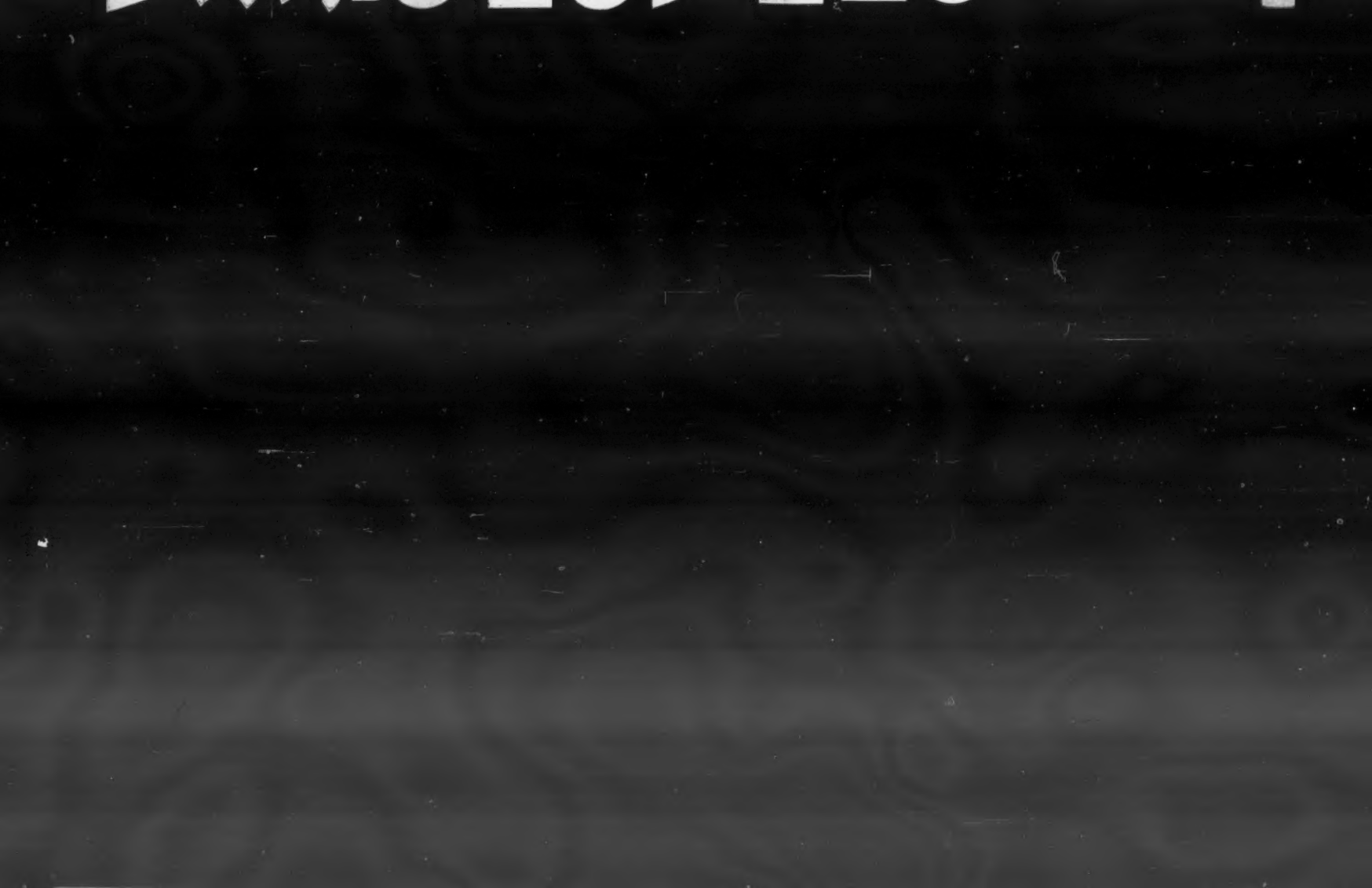
[ *Illustrations follow* ]

## LEGENDS FOR FIGURES

FIG. 1. Lungs, rabbit. External surfaces showing the quantitative differences in infarcts 3 days after a single intravenous injection of hexachlorotetrafluorobutane in doses of 0.05 ml. per kg. (left) and 0.40 ml. per kg. (right). × 2.

FIG. 2. Lungs, rabbit. Section of an infarct, 4 days old, showing, from right to left, the zone of coagulative necrosis, narrow boundary of cellular infiltration which separates non-viable from viable tissue, and the peripheral zone of incomplete (hemorrhagic) infarction. Hematoxylin and eosin stain. × 20.



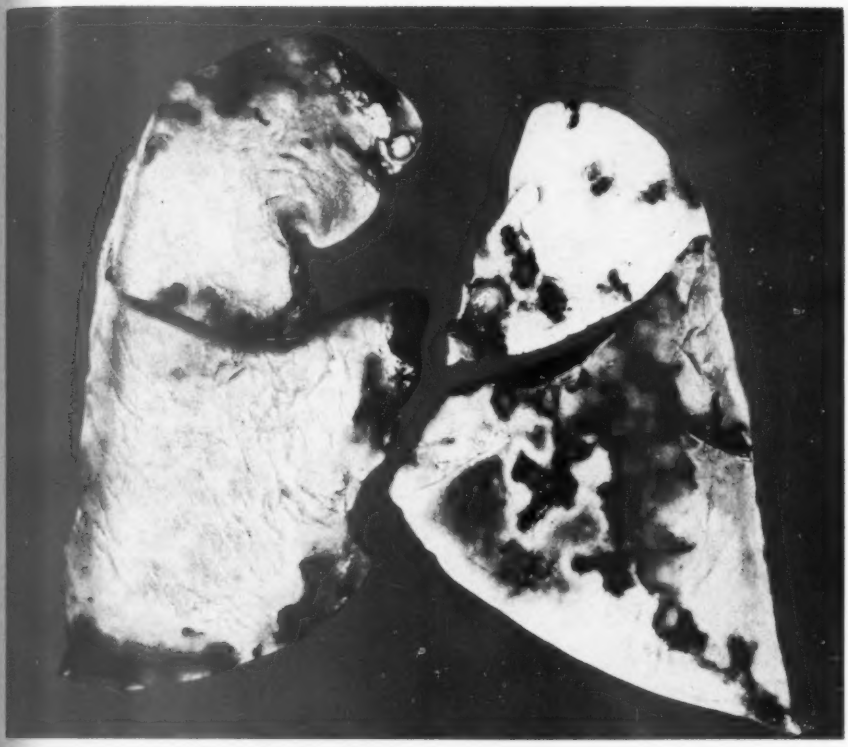


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FIG. 3. Lung, rabbit. Early infarction, 6 hours after 0.10 ml. per kg. of intravenous hydrocarbon. Vascular distribution of capillary hyperemia extending from a damaged arteriole. The larger arterioles are stuffed with leukocytes. Hematoxylin and eosin stain. × 85.

FIG. 4. Lung, rabbit. Healed infarction 3 months after 0.20 ml. per kg. of intravenous hydrocarbon. Peripheral scarring, emphysema, bronchiolar hyperplasia, and endarteritis obliterans may be noted. Intraluminal calcific deposits lie near the sclerosed artery. Hematoxylin and eosin stain. × 65.

FIGS. 5 to 7. Pulmonary arterioles, rabbit. Progressive endarteritis in vessels at the periphery of infarcts. (Fig. 5) Intimal alterations and perivascular telangiectasis 1 day after 0.10 ml. per kg. of intravenous hydrocarbon. Hematoxylin and eosin stain. × 215. (Fig. 6) Intimal proliferation, leukocytic infiltration, and perivascular edema 4 days after 0.10 ml. per kg. of intravenous hydrocarbon. Hematoxylin and eosin stain. × 120. (Fig. 7) Recanalized, fibrosed thrombus with intact elastic membrane 7 days after 0.10 ml. per kg. of intravenous hydrocarbon. Hematoxylin and eosin stain. × 250.

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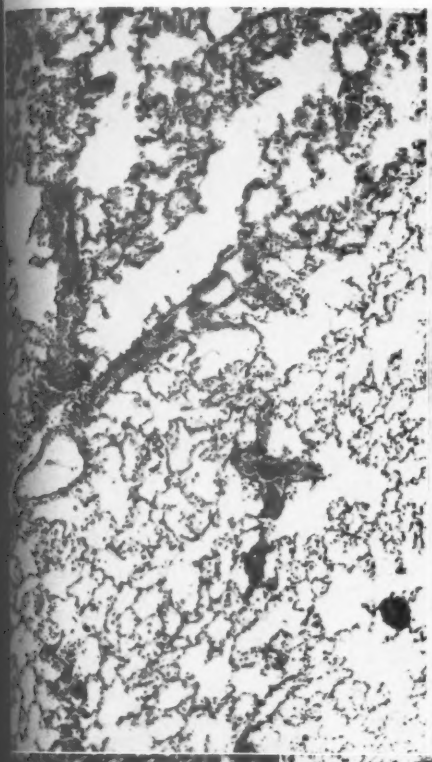

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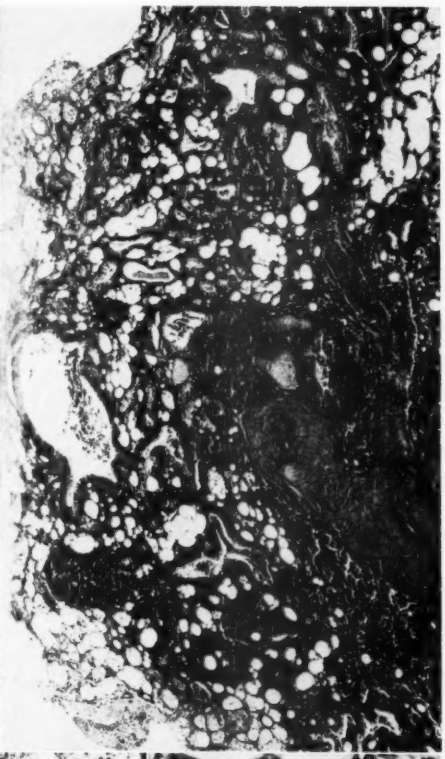

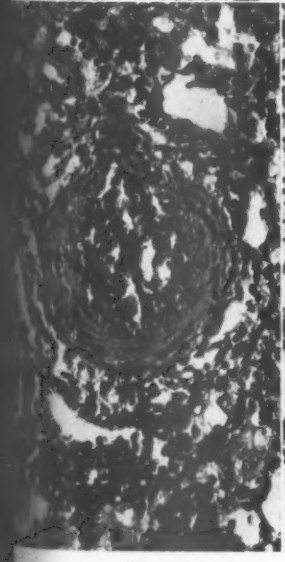

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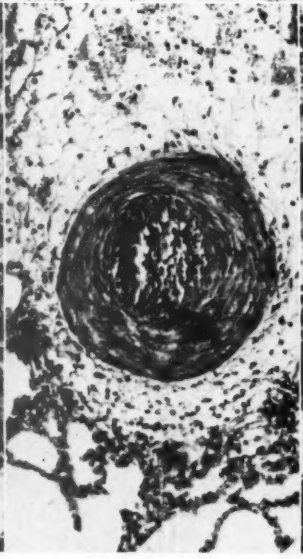

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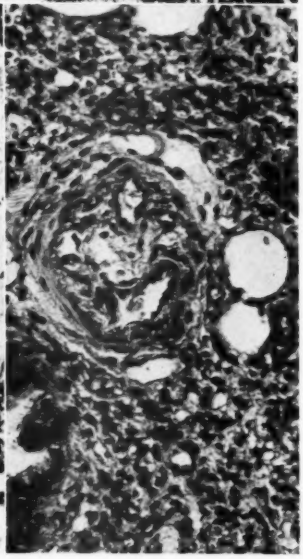

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FIG. 8. Lung, rabbit. Zone of hemorrhagic infarction 3 days after 0.40 ml. per kg. of intravenous hydrocarbon. Prominent hyaline membranes line the alveolar ducts and interstitial inflammation is extensive. Hematoxylin and eosin stain. × 120.

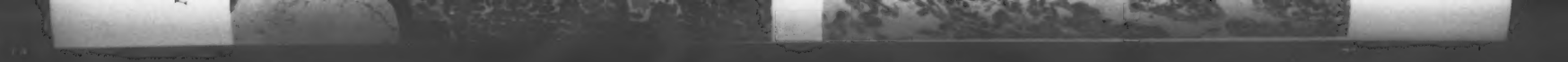
FIG. 9. Lung, rabbit. Healed infarct 3 months after 0.20 ml. per kg. of intravenous hydrocarbon. Disoriented elastic tissue with loss of alveolar pattern. Pleural surface upper right. Weigert's elastic tissue stain. × 100.

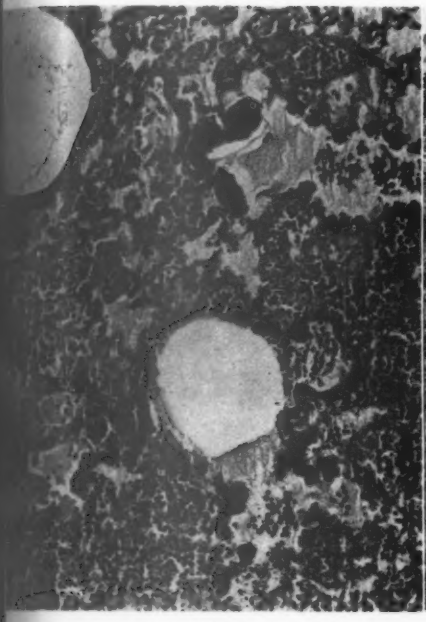
Figs. 10, 11, and 12. These photomicrographs illustrate the spectrum of epithelial hyperplasia bordering healed infarction.

FIG. 10. Lung, rabbit. One month after 0.20 ml. per kg. of intravenous hydrocarbon. Focus of alveolar fetalization in the vicinity of an infarct but separated from the scar by normal lung tissue. Hematoxylin and eosin stain. × 250.

FIG. 11. Lung, rabbit. Two months after 0.20 ml. per kg. of intravenous hydrocarbon. Bronchiolar hyperplasia and squamous metaplasia at the margin of a healing infarct. Hematoxylin and eosin stain. × 250.

# MEXICO





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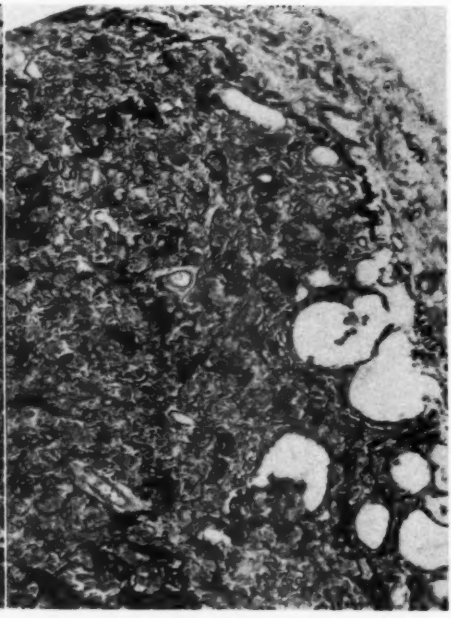

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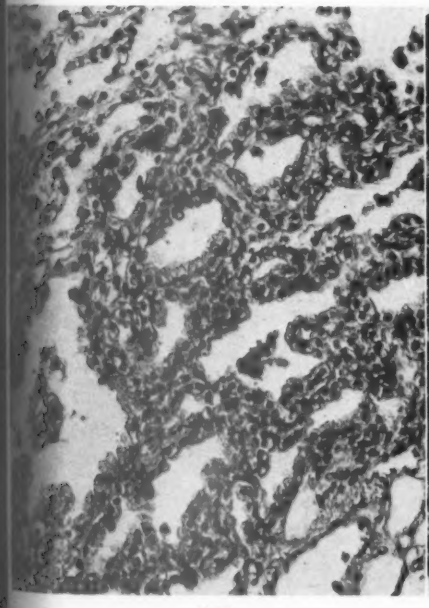

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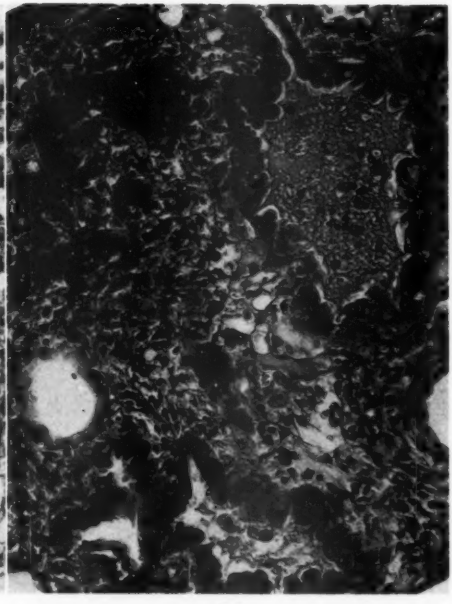

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FIG. 12. Lung, rabbit. Four and one-half months after 0.10 ml. per kg. of intravenous hydrocarbon. Atypical bronchial proliferation and hyperchromatism within an area of healed infarction. Pleural surface upper right. Masson's trichrome stain. × 50.

FIG. 13. Lung, rabbit. Phagocytosed calcium in a regional lymphoid nodule near the margin of a healed infarct, 3 months after 0.20 ml. per kg. of intravenous hydrocarbon. Alizarin red stain. × 120.

FIG. 14. Lung, rabbit. Ossifying nodule 3 months after 0.20 ml. per kg. of intravenous hydrocarbon. Transition from the calcific material to bone, the calcium being partially surrounded by respiratory epithelium. Hematoxylin and eosin stain. × 250.

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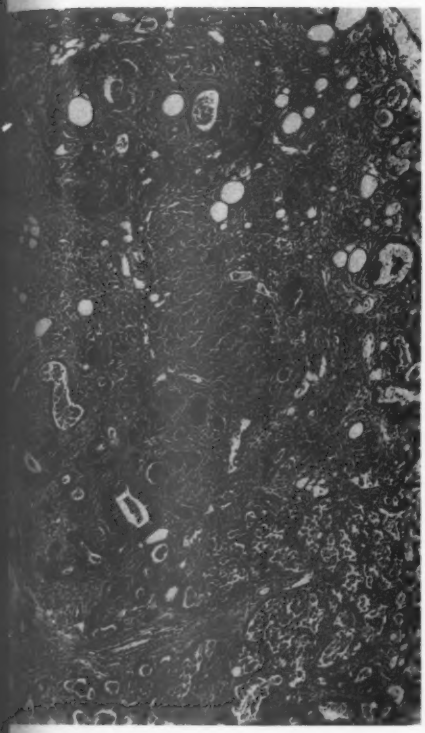

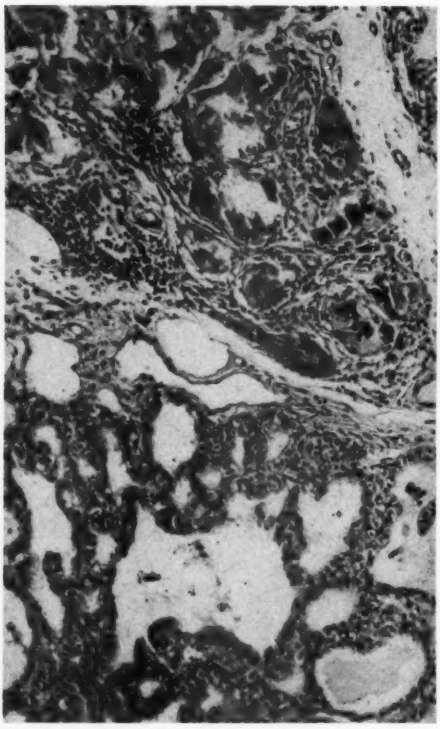

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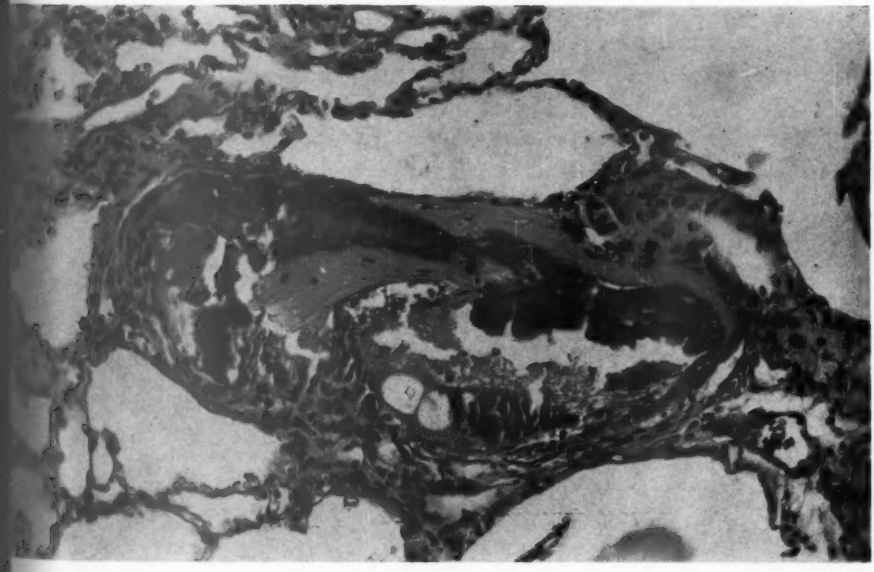

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# TUBERCULOSIS INDUCED BY DROPLET NUCLEI INFECTION

## Its Developmental Pattern in Guinea Pigs and Rats in Relation to Dietary Protein *

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Tuberculosis, as induced in mice, rats, hamsters, guinea pigs, and rabbits by droplet nuclei infection, passes through two distinct phases that apparently reflect stages in developing resistance. The first phase is completed within about 3 weeks. During this time the rate and pattern of tubercle formation are the same for each species of animal irrespective of the human or bovine origin of the *Bacillus tuberculosis* used to infect them. The second phase, which follows immediately upon the first, is distinctly different. In this phase the rate and pattern of tubercle formation quickly become characteristic of the host-parasite combination. Nevertheless, the rate and pattern of tubercle formation in any one host-parasite combination are highly uniform for a period, the duration of which differs with the combination.[1-4]

A considerable amount of evidence suggests that the level of dietary protein is a factor in developing resistance to tuberculosis.[5-10] Such a factor should become apparent during or soon after the second phase of the disease, especially when this is relatively prolonged. The following host-parasite combinations seemed to be the most convenient for the purposes of the present series of experiments to test this hypothesis: hamsters and guinea pigs with bacilli of human origin, and rats with bacilli of bovine origin.[3,4] The periods of uniform reaction with these combinations have ranged from about 50 days for the guinea pigs to more than 150 days for the rats.

The results of the experiments on hamsters have been published.[10] The present report describes the results of concurrent studies on guinea pigs and rats.

### Material and Methods

The three isocaloric diets used in these experiments have been found to be about equally acceptable to hamsters, rats, and guinea pigs, and to induce similar responses in these animals. These diets were desig-

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nated by the letters A, B, and C, and their respective composition was stated fully in the paper on hamsters.[10] Protein comprised approximately 30, 17, and 6 per cent of the respective diets. Ascorbic acid was added to the food for guinea pigs in amounts sufficient to supply about 20 mg. per day to each animal. In addition, each guinea pig was given about 25 gm. of kale per day.

Short-haired guinea pigs (300 gm.) and rats (100 gm.) of Long-Evans strain were purchased as immature animals. Animals of each sex were caged separately in small groups. Weights were recorded biweekly. A stock diet was fed until the guinea pigs attained weights of at least 500 gm. and the rats, 150 to 200 gm. Test diets were then fed for at least 6 weeks before infections were induced, at which time the weights of guinea pigs ranged from 550 to 800 gm., and of rats from 180 to 300 gm.

Infections were induced by means of the Wells apparatus and techniques.[11-13] A single aqueous suspension of tubercle bacilli was used to infect the groups of animals on each of the three diets. Thus all animals in any one experiment inhaled organisms of highly uniform virulence and viability.[8] Infections were induced at approximately minimal level, mean numbers of initial tubercles in animals of the several experimental groups ranging from four to eight.

Guinea pigs were infected by *B. tuberculosis* of the H37Rv strain, and rats by bacilli of the Ravenel strain. Cultures were supplied by the Standard Culture Depot of the National Tuberculosis Association, Trudeau, New York, and were maintained on solid and liquid media.[12]

During the experiments one culture of the H37Rv strain became reduced in virulence, but before this change was recognized both hamsters and guinea pigs had been infected with these organisms. The reaction of three diet-groups of hamsters to this organism have been described previously.[10] Following an account of the effects of the diets upon tuberculosis induced by the virulent bacilli of this strain, a description of the reactions of three diet-groups of guinea pigs to the attenuated variety will be given. Bacilli of the Ravenel strain did not change in virulence during this study, as measured by the rate of tubercle formation in rabbits.[14]

From 4 to 14 guinea pigs of each diet-group were sacrificed for study at intervals of from 50 to 180 days after infection; and, similarly, 3 to 12 rats from each group were sacrificed 75 to 260 days after infection. Tissues were prepared for study by the methods previously described for hamsters.[10]

## Tuberculosis of Guinea Pigs Induced by Bacilli of High Virulence

Significant differences in the appearances of the lesions in these animals became evident about 75 days after infection and increased rapidly thereafter. Deaths from tuberculosis occurred in animals on diet C within 140 days after infection, and none of the guinea pigs infected by the more virulent organisms was observed beyond 150 days.

### *Macroscopic Appearances*

Figures 1 and 2 illustrate the essential differences in tuberculosis of animals on diets A and C 100 days after infection. At this time the disease in animals on diet B corresponded closely to that in animals on diet A. Thus a majority of the initial tubercles of animals on diets A and B were well defined, solid nodules about 5 mm. in diameter, which was the size of the lesions at 50 days in guinea pigs of all diet-groups. In contrast, a majority of initial tubercles in animals on diet C were not well defined at 100 days, apparently having expanded irregularly from the spherical nodules found earlier. As a rule this change was associated with increased numbers of secondary tubercles in the lungs.

The tubercles of the thoracic lymph nodes usually enlarged between 75 and 100 days after infection, irrespective of the diet, but occasionally were no larger than at 50 days. Liquefaction had developed in many of these lesions within 100 days, irrespective of their size. This change usually was most prominent in animals on diet C. The tubercles in the spleens of animals on diet C sometimes increased in size between 75 and 100 days, whereas in animals on diets A and B, tubercles in this organ apparently regressed after 75 days, and sometimes disappeared within 100 days (Figs. 1 and 2). The livers did not contain macroscopic tubercles at this time.

At 150 days after infection some of the tubercles of animals on diets A and B still were as well circumscribed as at 50 days and had not changed in size. More often, however, the lesions were smaller and not so well defined. The tubercles of the thoracic lymph nodes of animals on these diets usually were smaller than at 100 days, and their spleens did not contain active tubercles. However, the livers of several of them contained small scars and occasional tubercles (Figs. 3 and 4).

In contrast to the state of the disease in animals on diets A and B, a majority of the animals on diet C had developed relatively large cavities, which were associated with extensive local secondary spread

of the infection in the lungs. In others of this group the disease was less extensive in the lungs, but apparently was progressing. Tubercles of the thoracic lymph nodes of the group on diet C ranged as widely in size as at 100 days and possessed conspicuous centers of liquefaction.

The livers of all animals on diet C were more or less distorted throughout by tiny scars which sometimes were associated with small hemorrhagic foci. However, macroscopic tubercles were not found in this organ, and were present in the spleens of only three of this group.

### *Microscopic Appearances*

Figure 7 illustrates the characteristic features of initial tubercles of animals of all diet-groups 50 days after infection, at which time the effects of diets had not become evident. Approximately the central half of each of the tubercles was necrotic and densely infiltrated by polymorphonuclear leukocytes. The periphery was formed by densely massed monocytes. These cells had infiltrated the alveolar walls and spaces to produce expanding zones of consolidation, and had accumulated cords and masses in perivascular and peribronchial lymph spaces to give irregular extensions to the lesions. Bacilli were demonstrated only among the partially degenerated monocytes in the necrotic centers of the tubercles. Usually no more than two or three organisms were found within the outlines of a cell at this time.

The state of the secondary tubercles in the thoracic lymph nodes corresponded closely to that of the initial lesions. The lungs, spleens, and livers of all animals also contained scattered, small, secondary tubercles. Usually these were circumscribed masses of monocytes.

About 75 days after infection differences in the histologic pattern of tuberculosis, that could be related to the diets, became evident. These differences increased with time and were developed most strikingly in the initial tubercles and their associated lesions in the lungs (Figs. 8, 10, 11, and 12).

One hundred days after infection the initial tubercles of guinea pigs on diet C were, to a large degree, merely expansions of those seen at 50 days. At the same time their perivascular and peribronchial extensions were increased in size, expanding into the surrounding alveolar tissues and through the walls and mucosa of bronchi and bronchioles (Figs. 8 and 12). Endovascular reactions were not conspicuous at this time.

Bacilli could be demonstrated throughout the necrotic portions of these tubercles at 100 days, but were most numerous among the fragmented leukocytes. In these sites the microorganisms, present in small, dense clumps, showed a distinct increase over the numbers that had

been found at 50 or 75 days. This evidence of unchecked expansion of lesions in the lungs and increased numbers of bacilli were not found at 75 days in animals on diet A, nor at 100 days in animals on either diet A or B.

At this time, the initial tubercles of all animals on diet A and of a majority on diet B were enclosed by fibrous tissue in which there were calcium deposits. Now, too, the peripheral extensions of these lesions about the blood vessels and bronchi were reduced to inconspicuous fibrous scars (Figs. 10 and 11). As a rule, the miliary tubercles seen at 50 days had disappeared from the lungs of these animals by 100 days, or were represented by scattered, small, dense clusters of monocytes and lymphocytes.

The necrotic centers of the initial tubercles of animals on diets A and B were occupied more or less completely by polymorphonuclear leukocytes, which seemed to be suspended in a faintly eosinophilic matrix. Apparently the tubercles in these animals had liquefied without bronchial spread of the infection. Bacilli still could be demonstrated among the leukocytes in the necrotic centers, but only in small numbers, and not in all lesions.

At 100 days, tubercles of thoracic lymph nodes of animals on diets A and B contained relatively small liquid centers, enclosed in loosely arranged granulation tissue, in which lymphocytes now formed conspicuous groups. Calcium deposits had formed in the granulation tissue adjacent to the necrotic centers. Small cellular tubercles were found in occasional spleens and livers of these animals.

In the guinea pigs on diet C at 100 days, tubercles of the thoracic lymph nodes contained relatively larger centers of liquefaction enclosed in densely cellular granulation tissue. Calcium deposits were not seen in these lesions.

Tubercles, usually appearing as solid cellular masses, were relatively numerous in the spleens and livers of animals on diet C at 100 days. In the liver the tubercles sometimes were associated with small infarcts. These apparently developed when the tubercles, frequently located in the portal regions of the lobules, encroached upon afferent blood vessels to cause angiitis.

One hundred and fifty days after infection the initial tubercles of animals on diets A and B ranged from solid fibrous masses that contained few inflammatory cells and scattered calcium deposits to sharply circumscribed, thick-walled spaces, the small, apparently liquid centers of which were occupied largely by polymorphonuclear leukocytes (Figs. 10 and 11). These centers were surrounded by zones of proliferating granulation tissue that contained calcium deposits. Exudate

from the liquefying centers was not found in adjacent bronchioles, nor were bacilli demonstrated in sections of these tubercles.

The structure of the more active tubercles of animals on diet A was reproduced fairly well in the corresponding lesions of two guinea pigs on diet C. However, in these instances, bacilli were relatively abundant among the cells of liquefying centers and among the dense masses of inflammatory cells, largely polymorphonuclear leukocytes, found within bronchioles near the tubercles. The only source of this exudate seemed to have been the centers of the tubercles, the walls of which were continuous with small but apparently expanding perivascular and peribronchial lesions.

The changes in the lungs of other animals on diet C were confused at this time. Cavities were continuous with relatively large segments of consolidated parenchyma that seemed to be direct extensions of the perivascular and peribronchial foci of infection. Evidence of endobronchial spread of the infection was uncertain, even in the presence of the obviously active cavities. The cavities and adjacent consolidated foci in three animals were continuous with partially calcified, fibrotic initial tubercles. These older lesions still contained active foci of infection and many bacilli, but had not liquefied. Evidently, cavity formation in these animals did not depend primarily upon liquefaction of the initial tubercles. The contribution of the tubercles to a cavity was not so great as that of the secondary lesions about them.

Large numbers of bacilli were found in all active foci of infection in the lungs of animals on diet C at 150 days, especially in foci of liquefaction.

The state of the secondary tubercles of the thoracic lymph nodes could not be related to diet at this time. Centers of liquefaction apparently had developed in all of them, but either they were undergoing fibrosis or had been replaced by fibrous tissue in which there were deposits of calcium. The spleens of only two animals on diet C contained small cellular tubercles. However, such lesions were present in the livers of all animals on diets B and C, and of one on diet A. Bacilli were demonstrated with difficulty in sections of the secondary tubercles in the livers and spleens of animals on diet C, but were not found in these lesions of animals on other diets.

## *Summary*

The development pattern of tuberculosis, induced in guinea pigs by inhalation of virulent bacilli of human origin, was not influenced by levels of protein in diets containing 30, 17, and 6 per cent, respectively,

for about 75 days. Beyond this time the effects of dietary protein were evident chiefly in the lesions of the lungs, which continued to progress only in the animals receiving 6 per cent protein. Development and regression of secondary lesions were not so clearly related to the diets.

### Tuberculosis of Guinea Pigs Induced by Bacilli of Lower Virulence

Three diet-groups of 25 to 31 guinea pigs were infected, and several from each group were killed 75, 125, and 180 days later. Counts of initial tubercles at 75 and 125 days indicated that guinea pigs were quite as susceptible to infection by these organisms as to the more virulent bacilli. However, the size of initial tubercles and the state of the infection at 125 days corresponded approximately to those found 50 days after infection by the more highly virulent bacilli.

Figures 5 and 6 illustrate the stages of lesions found in animals on diets A and C 180 days after infection. At this time the infection seemed to be about equally advanced in the lungs of all animals, irrespective of the diet. Outlines of initial tubercles were ill defined and continuous with irregular peribronchial and perivascular lesions. In addition, the lungs of many animals contained large numbers of apparently isolated, secondary tubercles.

Secondary tubercles of the thoracic lymph nodes were advanced about equally in all diet-groups at 180 days, and in approximately the state found at 100 days after infection by the more virulent bacilli. The livers of all animals were free of lesions, as were the spleens of all animals on diets A and B. In five of the ten animals on diet C, the spleens were enlarged, although macroscopic tubercles were not recognized.

Microscopic examination disclosed that the state of these initial tubercles 75 days after infection was closely similar to the stages found at 50 days in guinea pigs that had inhaled the more virulent bacilli. Between 75 and 125 days the necrotic centers of a majority of the tubercles became calcified and surrounded by fibrous tissue. At the same time the outer borders of the tubercles apparently continued to expand irregularly (Fig. 9) and 180 days after infection the appearances of the initial tubercles suggested that the irregular peripheral expansion had continued much the same in each of the diet-groups. However, ulcers in the bronchi were always small, and seemed to be relatively inactive.

Tubercles in the thoracic lymph nodes of animals on diets A and B had progressed to partial liquefaction and focal calcification within

125 days, and by 180 days had been replaced by dense fibrous tissue. Liquefaction seemed to have occurred later and to a greater extent in the thoracic lymph nodes of animals on diet C which, at 180 days, contained relatively larger centers of liquefaction.

In animals on diets A and B, tubercles in the spleen had been replaced by fibrous tissue within 125 days, and in one half of those on diet C this occurred within 180 days. In others of this group the spleen was found to have been irregularly expanded by rather large collections of epithelioid cells in which one or two bacilli per cell were sometimes demonstrated. In the other foci of infection, bacilli were difficult to demonstrate until 180 days when their numbers were about the same in animals of all diet-groups, and about equal to the numbers found at 50 days in the initial tubercles of guinea pigs that had inhaled the more virulent bacilli.

*Summary*

The developmental pattern of tuberculosis, induced in guinea pigs by the inhalation of attenuated bacilli of human origin, was not influenced by levels of dietary protein for about 100 days. Beyond this time the effects of dietary protein were evident chiefly in the secondary lesions of thoracic lymph nodes and spleen which, in animals on the higher levels of dietary protein (17 to 30 per cent) began regression within 125 days. Tuberculosis of the lungs continued slowly progressive, irrespective of the diets, throughout the 180 days of the experiment.

## Tuberculosis of Rats

Levels of dietary protein neither influenced the susceptibility of rats to infection by bacilli of the Ravenel strain nor modified the progress of the initial tubercles of these animals within 260 days. Secondary tubercles did not develop to macroscopic size.

Microscopic examinations demonstrated that the initial tubercles of rats retained one form throughout the period of the study. This form was established within 5 weeks after infection and changed neither with time nor with the level of dietary protein.

These lesions were irregularly outlined foci in the lungs, in which the interstitial tissues were thickened by accumulations of lymphocytes and monocytes. Monocytes also accumulated in the alveolar walls and spaces within the foci, but the most conspicuous cells of the initial tubercles of rats were much larger and more lightly stained, and their cytoplasm was finely vacuolated. A majority of the cells were uninucleate but occasional ones contained two to four nuclei. Probably they

should be called monocytes too, but monocytes that had undergone a change after having ingested bacilli, for these were the only cells in which bacilli were found. However, the number of bacilli even at 260 days was never more than four to six per cell.

With the exception of a brief episode during the third week, the tubercles induced in rats by inhalation of droplet nuclei have not been found to undergo necrosis. Peripheries never were limited by fibrous tissue, and enlargement always seemed to have been across alveolar walls.

Secondary tubercles were found only in the thoracic lymph nodes and at no time were larger than a mass of about ten of the large vacuolated phagocytes, closely pressed together. Such lesions were present in small numbers in rats of all groups at 75 days, and continued so through 260 days.

## Discussion

These experiments demonstrate that levels of dietary protein can be critical factors in the development of resistance to tuberculosis. This demonstration was possible only when infections were induced by bacilli that were moderately virulent for the animals. For example, bovine bacilli of the Ravenel strain, which are highly virulent for hamsters and guinea pigs, overwhelm the animals so rapidly that effective resistance cannot develop.[4] Conversely, when hamsters and guinea pigs were infected by relatively avirulent bacilli, or rats infected by bacilli of the Ravenel strain, levels of dietary protein had no demonstrable effects upon the pattern of tuberculosis (especially in the lungs) within the periods of the experiments. The evidence suggests that these organisms grew so slowly in the lesions that antigens were inadequate for the production of effective levels of antibodies.[10]

Failure to demonstrate an effect of protein and of other dietary factors upon the development of resistance to tuberculosis has been reported many times.[15] The present series of experiments suggest that these failures may reflect the choice of an unsuitable host-parasite combination, composition of the diets, or length of observation period.

For example, Metcoff and associates[15] compared the effects of protein at levels of 2, 8, and 18 per cent upon tuberculosis of rats during a period of 58 days following intravenous inoculation. The results were negative, as those of the present experiments on guinea pigs would have been, had observations ended within 58 days. On the other hand, Koerner, Getz, and Long[16] found distinctive differences in the patterns of tuberculosis in rats when protein constituted 15 to

40 per cent of the diet. Here, too, infections were induced by intravenous injection of relatively large numbers of bacilli. Differences, however, did not become evident until the disease had developed for about 150 days. This suggests that if the rats in the present experiments had inhaled much larger numbers of bacilli, the disease might have been altered by the diets. However, inhalation of only a small fraction of the number of bacilli estimated to have been injected into these rats probably would have led to death within 4 to 5 weeks, as the developing tubercles expanded to fill the lungs.[1,2] Thus the rat is not a suitable host for studies of the relation of nutrition to tuberculosis induced by inhalation. The mouse probably is little better.[4] The results of these studies on rats, however, support the opinion that in tuberculosis, resistance develops as a function of time and the growth rate of the bacilli, i.e., the rate at which antigen becomes available.[10] The development of resistance also must depend upon adequate levels of dietary protein,[17] which may differ with the species of animal. In this connection it should be noted that on 17 per cent protein hamsters usually failed to develop evidence of resistance, while guinea pigs on this level developed resistance almost as rapidly and effectively as on 30 per cent protein.

## Summary and Conclusions

Six or more weeks before infection, groups of young adult guinea pigs and rats were started on isocaloric diets containing approximately 30, 17, and 6 per cent protein, and were continued on these diets until they were sacrificed for study at stated intervals from 50 to 260 days after infection. Tuberculosis was induced by inhalation at approximately minimal levels. Guinea pigs were infected by bacilli of human origin, and two strains with different levels of virulence were used. Rats were infected by bovine bacilli of the Ravenel strain.

Levels of dietary protein did not influence susceptibility of either rats or guinea pigs to infection by inhaled bacilli nor modify the progress of tuberculosis of rats within 260 days. In guinea pigs which inhaled the less virulent bacilli the developmental pattern of pulmonary lesions did not change within 180 days. However, secondary lesions at sites beyond the lungs healed more rapidly in animals on the higher levels of dietary protein.

When guinea pigs inhaled the more highly virulent bacilli the progress of the disease was not influenced by the diets within 50 days. At 75 days and beyond, all lesions in guinea pigs on the higher levels of die-

tary protein seemed to be healing, and even in animals on 6 per cent protein only the lesions in the lungs continued to be active.

It is concluded that the level of dietary protein can be a critical factor in the development by guinea pigs of effective resistance to tuberculosis. This requires relatively prolonged interaction of host and virulent parasite, and apparently reflects the combined actions of monocytes and other factors which, presumably, are antibodies.

It is concluded also that the highly uniform response of guinea pigs for upward of 50 days after infection is further evidence against the concept of native or hereditary resistance.

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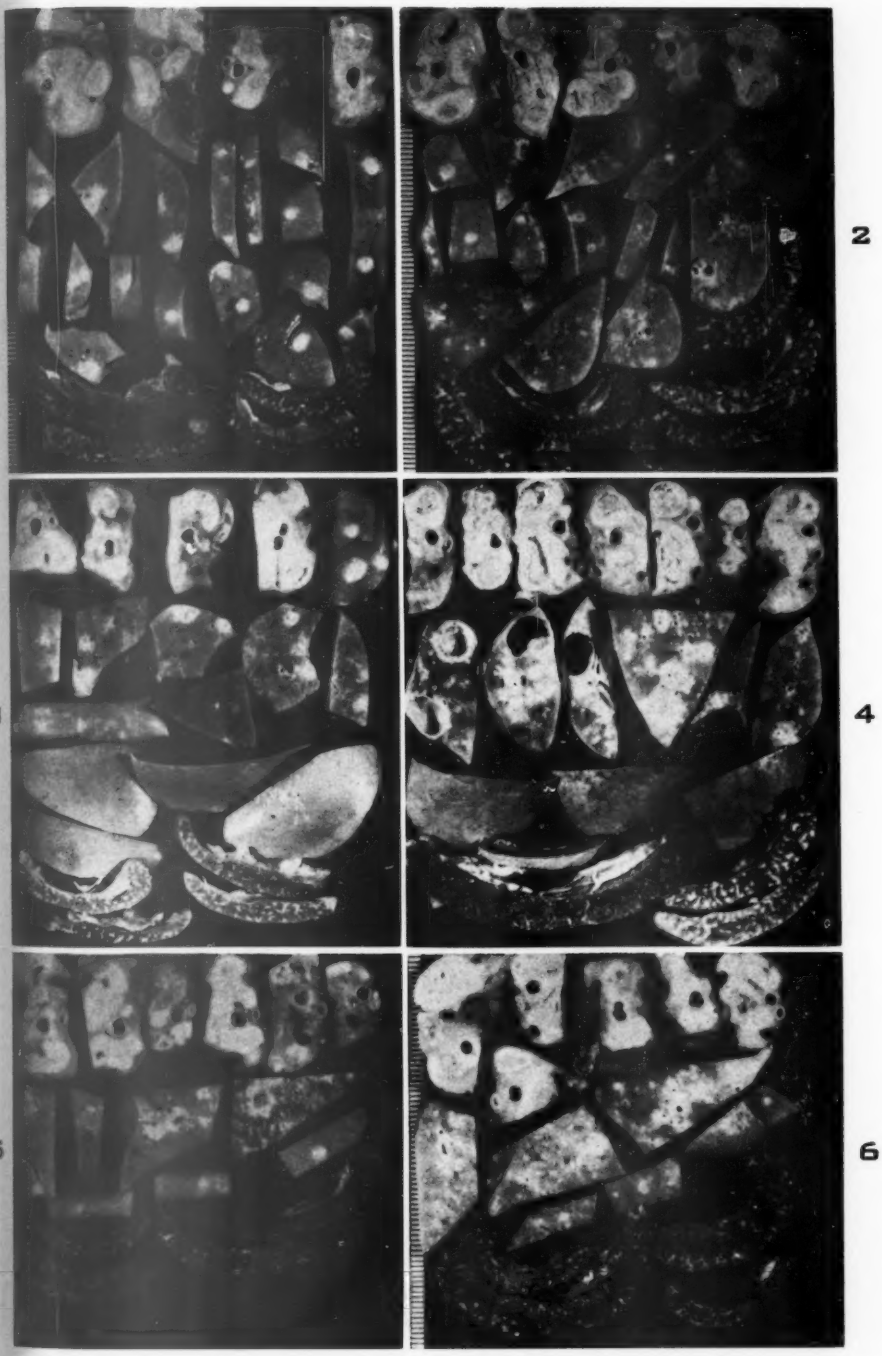
## LEGENDS FOR FIGURES

FIGS. 1 (diet A) and 2 (diet C) from guinea pigs 100 days after infection with bacilli of high virulence. Specimens in photographs are arranged in the following order: *upper*—thoracic lymph nodes cut vertically to long axis of body and including sections of trachea, esophagus, and aorta with the nodes; *center*—segments of the lungs of each animal; *lower*—longitudinal sections through spleens. In animals on diet A, tubercles in thoracic lymph nodes and lungs were undergoing fibrosis and tubercles in the spleens were completely fibrotic at this time. In animals on diet C, the thoracic lymph nodes contained large semiliquid foci. Tubercles in the lungs were poorly circumscribed, and secondary tubercles were abundant in the spleens.

FIGS. 3 (diet A) and 4 (diet C) from guinea pigs 150 days after infection with bacilli of high virulence. Specimens arranged as in Figures 1 and 2, but with bits of the free edge of the liver between lungs and spleens. Differences that may be attributed to the development of resistance on diet A and failing resistance on diet C are clearly evident in the lungs and lymph nodes. The enlarged spleen in Figure 4 was associated with extensive scarring in the liver; it was not tuberculous.

FIGS. 5 (diet A) and 6 (diet C) from guinea pigs 180 days after infection with bacilli of low virulence; for comparison with Figures 3 and 4. Lesions of the lymph nodes and spleens of animals on diet A seemed to be healing at this time, but apparently were progressive in the lungs of all animals, and in the spleens of about half on diet C.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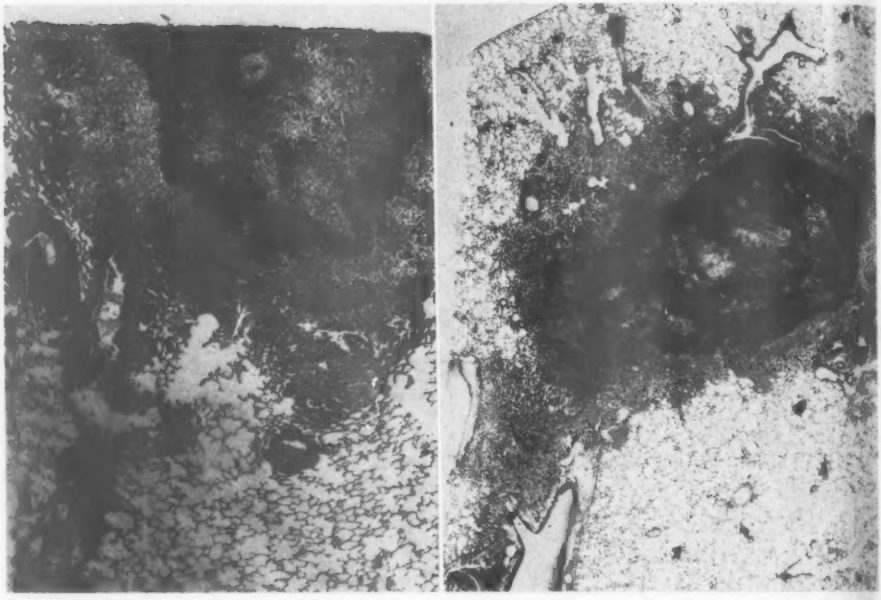


FIG. 7. Initial tubercle of guinea pig 50 days after infection by the more virulent bacilli. The upper half of the lesion was trimmed away, then sectioned at right angles to this cut. The extent of the necrotic center of the tubercle, the irregular periphery, and the peribronchial and perivascular extensions may be noted. Hematoxylin and eosin stain. × 30.

FIG. 8. Initial tubercle of guinea pig on diet C, 75 days after infection by the more virulent bacilli. Of note are the irregular peripheral extension, and exudate in a bronchus adjacent to the necrotic center, and irregular expansion of the necrotic center. Hematoxylin and eosin stain. × 10.

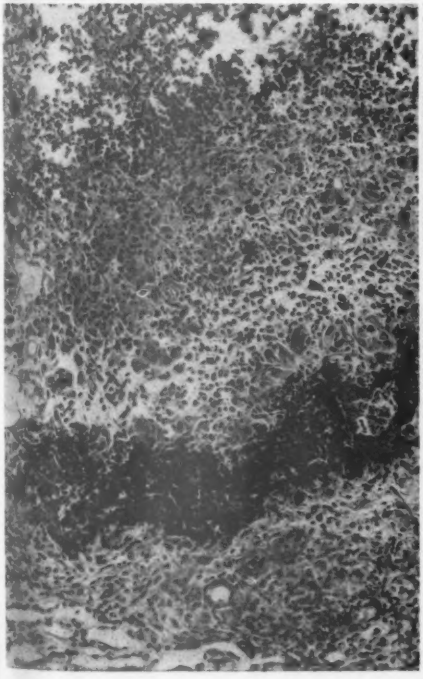
FIG. 9. Initial tubercle of a guinea pig on diet A, 125 days after infection by bacilli of low virulence. A sharply defined necrotic center with dark (calcified) masses is surrounded by a zone of granulation tissue. The periphery is irregular and, in the upper left, has ulcerated into a bronchus. Hematoxylin and eosin stain. × 12.

FIG. 10. Initial tubercle of a guinea pig on diet A, 150 days after infection by bacilli of high virulence. Of note are the remnants of liquefied material, upper left, the well developed, partially calcified fibrous tissue of the wall, and absence of disease in the wall of the adjacent bronchus. Hematoxylin and eosin stain. × 30.

FIG. 11. A segment of the wall of an initial tubercle of a guinea pig on diet A, 100 days after infection by bacilli of high virulence. A thin wall of fibrous tissue outlines this lesion. There is a zone of calcium, with inflammatory cells within this zone. Hematoxylin and eosin stain. × 120.

FIG. 12. A part of a peribronchial extension of an initial tubercle of a guinea pig on diet C, 100 days after infection by bacilli of high virulence. An eccentric mass of tuberculous granulation tissue occupies the wall and replaces the mucosa. Hematoxylin and eosin stain. × 120.

10



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# BRONCHIOLAR EMPHYSEMA

## So-called Muscular Cirrhosis of the Lungs *

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Diffuse vesicular emphysema is a common and well recognized clinical and pathologic entity. It was first observed at necropsy by Baillie[1] as early as 1793. Since the classical descriptions by Laennec[2] in 1819 and Rokitansky[3] in 1842, little has been added to the understanding of its pathogenetic and morphologic features. Recently, we encountered two examples of microcystic disease of the lungs which, because of certain anatomical as well as clinical manifestations, warrant their consideration as an unusual type of emphysema. The clinical features of progressive dyspnea, chronic unproductive cough, and cor pulmonale were outstanding. Grossly, the involved portions of the lungs had finely bosselated and fibrous external surfaces, resembling the appearance of the liver in Laennec's cirrhosis. The cut surfaces presented numerous small air-containing cysts measuring from 1 to 3 mm. in diameter. These were interposed among dense and apparently imperfectly aerated pulmonary tissue. Microscopically, the salient features were marked dilatation of the respiratory bronchioles and hypertrophy of their muscular coats. The alveoli were diminished, inconspicuous, or absent, and areas of fibrosis were frequent. A comprehensive review of the literature revealed only ten cases (Table I) designated by various terms which appeared sufficiently similar and documented to be considered examples of this lesion. Only two have been recorded in the American literature.[4]

The first detailed report of this pulmonary disease was made by Rindfleisch[5] in 1897. He was obviously impressed by the gross appearance of the lungs, for he designated the condition cystic cirrhosis of the lungs. It is noteworthy that Virchow and Ziegler, in discussing this case, considered it to represent a special or peculiar form of emphysema. Several years previously, von Buhl[6] had employed the designation muscular cirrhosis of the lungs when muscular hypertrophy of the smallest bronchioles and obliteration of alveoli occurred. He had attributed these changes to a chronic inflammatory process.

Davidsohn,[7] von Stössel,[8] Calma,[9] and Rubenstein and associates[4] subsequently recorded cases with similar clinical histories and apparently identical pulmonary lesions. Von Stössel studied his first patient

* Received for publication, March 16, 1957.

TABLE I

*Summary of Reported Cases of Bronchiolar Emphysema*

| Author | Date | Cases | Age | Sex | Term used | Spontaneous pneumothorax | Cor pulmonale | Distribution of lesions |
|---|---|---|---|---|---|---|---|---|
| Rindfleisch[5] | 1897 | 1 | 40 | M | Cirrhosis cystica pulmonum | No | Yes | Bilateral, diffuse |
| Pepere[10] | 1906 | 1 | 16 | F | Cystic degeneration of lungs | No | Yes | Bilateral |
| Davidsohn[7] | 1907 | 1 | 30 | M | Muscular cirrhosis of lungs | No | Yes, with acute endocarditis of pulmonic valve | Bilateral |
| Loeschcke[11] | 1926 | 1 | 1½ | ? | Emphysema bronchiolectaticum | No | ? | Bilateral, slightly more marked at margins |
| von Stössel[8] | 1937 | 2 | 51 | M | Muscular cirrhosis of lungs | Yes, left side | Yes | Bilateral, more marked at margins; vesicular emphysema at apices |
| | | | 43 | F | Muscular cirrhosis of lungs | Yes, left side | Yes | Bilateral, most marked in right lower lobe |
| Romhányi and Maccone[12] | 1937 | 1 | 16 | M | Honeycomb lungs | Yes, right side | Yes | Bilateral, more marked in lower lobes and at margins |
| Calma[9] | 1941 | 1 | 36 | M | Cystic emphysema | No | Yes | Bilateral, more marked at margins; mixed with vesicular emphysema in upper lobes |
| Rubenstein, Gutstein, and Lepow[4] | 1955 | 2 | 63 | F | Pulmonary muscular hyperplasia | No | Yes | Bilateral, more marked in right lung; mixed with vesicular emphysema in left lung |
| | | | 65 | M | Pulmonary muscular hyperplasia | No | ? | Bilateral, more marked in lower lobes and margins |
| Siebert and Fisher | 1957 | 2 | 61 | M | Bronchiolar emphysema | No | Yes | Bilateral, more marked in lower lobes and margins; mixed with vesicular emphysema at apices |
| | | | 65 | M | Bronchiolar emphysema | No | Yes | Bilateral, diffuse |

for 7 years and commented upon the progressive increase of small, flecked opacities in a series of roentgenograms of the chest during that period. The descriptions of the gross and microscopic features of the lungs by these authors were essentially the same as those recounted by Rindfleisch, but, in addition, they observed thick or hypertrophied bands of smooth muscle intimately surrounding the cysts and associated with broken clumps and coils of elastic fibers. Von Stössel and Rubenstein *et al.* believed that the cysts were derived from small bronchioles by ectasia, but made no further identification of these structures. All these observers[4,7-9] noted also that there were reduced numbers of alveoli with thick septa, and in some areas no alveolar ducts were present. However, only Davidsohn remarked that the elastic lamellae of the alveolar walls were thickened. Calma, alone, interpreted his findings as being a manifestation of emphysema.

The cases of Pepere,[10] Loeschcke,[11] and Romhányi and Maccone[12] are included as examples in this review, despite the absence of muscular hypertrophy, because of the pathogenetic implications which may be derived from an analysis of their reports. The possibility that the absence of hypertrophy of smooth muscle might be attributed to the relatively short duration of the disease in these reports of an infant and two adolescents merits their inclusion with other examples of this pulmonary lesion.

In a different category are the many other isolated accounts of cases of cystic disease of the lung which, in many instances, may represent examples of bronchiolar emphysema. However, lack of adequate histologic description prevents absolute identification. In this group we place the fifth case of Buchmann[13] and that of Stewart and associates,[14] both of which occurred in females. In addition, seven cases of a condition described as pulmonary myomatosis have been reported.[15-21] These probably represent a similar lesion, although the authors did not comment upon the anatomical units involved or other microscopic details. Peculiarly, all of these patients also were female, and about half of them had some manifestation of tuberous sclerosis.

It becomes apparent that the nature of this unusual pulmonary disease has not been resolved, and a consistent demonstration of the precise anatomical units involved has not been made. It is the purpose of this article to record two additional examples of this condition, to indicate the relationship to emphysema, and to attempt to define the basic microscopic structure as well as the possible etiology and pathogenesis.

## Report of Cases

### *Case 1*

A Negro policeman, 61 years old, was first admitted to the hospital in 1949 because of excessive fluid in the left knee joint. At that time a history of chronic unproductive cough and wheezing at night for almost his entire life was elicited. He had had pneumonia in 1918 and 1944, although he was not hospitalized. He had previously smoked two packs of cigarettes a week, but had not used tobacco for several years because of asthma. He had experienced moderate dyspnea but not hemoptysis.

Physical examination revealed a barrel-shaped chest. Crepitant râles were heard at both lung bases. The chest was hyperresonant throughout. A pulmonic systolic murmur was heard. A blood count revealed 4,450,000 red blood cells and 6,200 white blood cells per cmm. The hemoglobin was 13.5 gm. per 100 ml. of blood and a differential white blood count was normal. Urinalysis and serologic tests for syphilis were negative. The total serum protein was 8.1 gm. per 100 ml. with 4.7 gm. of albumin and 3.4 gm. of globulin. The vital capacity was only 2.2 l., or 40 per cent of normal. Repeated sputum examinations and gastric washings were negative for acid-fast bacilli. Tuberculin, histoplasmin, and coccidioidin skin tests were consistently negative. Roentgenograms of the chest revealed marked pulmonary fibrosis, especially at the right base, and fine nodular infiltrations throughout both lung fields, most marked in the bases. Some cystic changes were noted in both upper lung fields. The patient received symptomatic therapy and was discharged.

He was re-admitted to the hospital 5 years later complaining of severe shortness of breath and cough. Physical examination and laboratory tests were essentially the same as on the previous admission. However, it was noted that his dyspnea had increased in severity. Roentgenograms of the chest revealed that the densities observed in 1949 had increased and that there was some confluence at the bases. No findings of significance were found at bronchoscopy. A clinical diagnosis of hypertrophic emphysema was made. Shortly before death his dyspnea became more marked and he complained of chest pain. Respirations became shallow and rapid, and the symptoms were not relieved by oxygen administration. He expired on November 26, 1954.

*Necropsy Findings.* The body was that of an elderly, obese Negro male. The chest was barrel-shaped and the abdomen slightly protuberant. The pleural cavities were free of fluid. There was no pneumothorax. The heart weighed 450 gm. The right ventricle was hypertrophied and dilated; the myocardium measured 6 mm. in thickness. The ring of the pulmonic valve was dilated and had a circumferential measurement of 7 cm. The left ventricular myocardium measured 13 mm. in thickness and the chamber was not appreciably dilated.

The lungs together weighed 1,550 gm. There were fibrous interlobar adhesions bilaterally, but no adhesions to the parietal pleura. The visceral pleura was uniformly studded by a myriad of miliary protuberances which gave the lungs an appearance similar to the liver of Laennec's cirrhosis (Fig. 1). The peripheries of the upper lobes presented emphysematous blebs, the lower lateral margin of the right

upper lobe bearing a large projecting and collapsible bleb measuring 2 cm. in diameter. The lungs were crepitant throughout, except for a firm reddened area at the base of the right lower lobe which on section proved to be a recent infarct. The branch of the pulmonary artery supplying this area contained a thrombus, but the remaining vessels were patent. Very little mucoid secretion was found in the bronchial tree and all the large bronchi showed no changes. The hilar lymph nodes were small and black. The cut surfaces of the lungs were moist, congested, and presented many spheroidal, cystic lesions which measured from 1 to 2 mm. in diameter. They were scattered diffusely throughout the substance of both lungs, but were more numerous in the lower lobes and in subpleural areas. These minute air-containing cysts were considerably fewer in the upper lobes and centrally.

### *Case 2*

A white male, 65 years old, who had been a bituminous coal miner for more than 30 years, was admitted to the hospital in 1955. He complained of progressively severe dyspnea and a chronic non-productive cough of 3 years' duration. There was no hemoptysis.

Physical examination revealed cyanosis of the nailbeds, ears, oral mucosa, and tongue. "Parrot-beaking" of the fingernails was noted, but there was no clubbing of the fingers. Severe dyspnea was present and respirations were shallow and rapid. The anterior-posterior diameter of the chest was observed to be increased. Hyperresonance and crackling râles were found throughout both lung fields. Occasional extrasystoles were noted and the pulmonic second sound was accentuated. The electrocardiogram showed evidence of right ventricular hypertrophy. A roentgenogram of the chest indicated a generalized interstitial pulmonary fibrosis. The patient was considered to be too ill for the determination of his vital capacity. Sputum examinations were negative for acid-fast bacilli. Repeated blood counts revealed a polycythemia with 5,900,000 to 5,200,000 red blood cells per cmm. and the hemoglobin was 16 to 15.5 gm. per 100 ml. of blood. The hematocrit varied from 60 to 53 per cent on successive determinations. The $CO_2$-combining power was 56 volumes per cent and serum chlorides, 90 mEq./l.

Clinical diagnoses of pulmonary emphysema with silicosis, cor pulmonale with paroxysmal atrial fibrillation and ventricular premature contractions, and secondary polycythemia were made. The patient was treated with bed rest, oxygen administration, and repeated phlebotomy. He was discharged from the hospital for a brief period, but was re-admitted in March, 1956, because of increasing dyspnea. At that time all laboratory studies were essentially the same as on his previous admission. The serum chlorides were 87.5 mEq./l. and the non-protein nitrogen 37 mg. per cent. Because of a total serum protein of 5.7 gm. per 100 ml. with 3.3 gm. of albumin and 2.4 gm. of globulin, phlebotomy was discontinued. He was treated with oxygen, but the dyspnea became more severe. The patient deteriorated steadily and expired on May 15, 1956.

*Necropsy Findings.* The body was that of a somewhat emaciated elderly white male. The anterior-posterior diameter of the chest was increased. The pleural cavities contained a few fibrous adhesions. The

peritoneal cavity contained approximately 1,500 cc. of clear, straw-colored fluid. The heart weighed 500 gm. The right ventricle was markedly dilated and hypertrophied, and extended caudad so as to form a greater portion of the apex than did the left ventricle. The myocardium of the right ventricle measured 7 mm. in thickness and that of the left, 12 mm. The ring of the pulmonic valve was dilated to a circumferential measurement of 9 cm. Significant atherosclerosis of the coronary arteries was absent. The liver weighed 1,600 gm. and exhibited a diffuse reddish brown discoloration, but no cirrhosis. Grossly, the pancreas, kidneys, and bone marrow were not remarkable. The spleen weighed 200 gm., presented a few small, recent and old infarcts, and the follicular markings were indistinct.

The lungs together weighed 1,800 gm. There were no discernible abnormalities of the trachea, major bronchi, or regional lymph nodes, although small quantities of mucoid secretion and slight mucosal injection were found in the secondary and smaller bronchi. A few scattered fibrous adhesions were found on the visceral and interlobar pleural surfaces of the lungs. The pleural surfaces of all lobes were finely bosselated with numerous small semispheroid projections resembling the surface of the liver of Laennec's cirrhosis. This change was only slightly greater in the lower lobes. A few small blebs were found at the apices. There was a slight decrease in crepitation of all lung tissue. The cut surfaces of all lobes revealed a multitude of miliary cystic cavities, measuring from 1 to 3 mm. in diameter, diffusely scattered throughout the entire pulmonary substance. These small cysts seemed to represent dilatations of the smallest bronchioles and were only slightly more numerous in the lower lobes and marginally than in the upper lobes or centrally. Their distribution was decidedly more diffuse than in case 1. A few larger air-containing cysts, which were essentially emphysematous blebs and measured 4 to 10 mm. in diameter, were found in the apices. The remaining pulmonary tissue showed a diffuse increase of fibrous connective tissue and appeared dense and red.

Microscopically, the liver showed periportal fibrosis and bile duct proliferation, but no perilobular fibrosis characteristic of cirrhosis. Rather large quantities of iron-positive brownish pigment were found in the hepatic, Kupffer, and bile duct epithelial cells, and to a much less degree in both the acinar and islet cells of the pancreas. These findings were considered indicative of incipient hemochromatosis. The bone marrow showed multifocal areas of erythroid hyperplasia charac-

teristic of a secondary polycythemia rather than of a polycythemia vera. Foci of extramedullary hematopoiesis were noted in the spleen and adrenal glands.

## Terminology

Considerable controversy has taken place concerning the anatomical constituency and significance of the respiratory unit first proposed by Rindfleisch[22] for which he used the now obsolete term pulmonary acinus.[23-27] We believe that the respiratory unit is a valid concept if redefined on the basis of modern morphologic and physiologic knowledge. The *respiratory unit* of the lung is a conical, porous or sieve-like structure which undergoes increasing alveolization as its base is approached, and is composed of a single respiratory bronchiole of the first order with all of its lateral and peripheral ramifications. Through all parts of the unit inspired air passes freely and to a less degree diffuses into neighboring units. We have adopted the term *cribellus* (Latin, "little sieve") as appropriate for this structure, and shall use it synonymously. Each respiratory unit or cribellus is divided ontogenetically, anatomically, and physiologically into two homologous but unequal parts. At its vertex lies its smaller *proximal* or *bronchiolar segment* (or, in biogenetic terms, the *archicribellus*), which is a vestigial and more primitive division and is nearly completely lined by epithelium. It consists of the respiratory bronchiole, stems of the second order, and the attached rudimentary alveoli. At the bases of the respiratory unit is found the *distal* or *ductal segment* (or *neocribellus*), which is by far the larger and more specialized portion. It is normally anepithelial, and is composed of the derived alveolar ducts and their numerous peripheral alveoli. Both segments are physiologically functional with regard to collateral air circulation, but by far the greater burden of hemo-gaseous exchange depends upon the distal, anepithelial portion where the alveolar capillaries are in more intimate contact with inspired air.

### *Method of Morphologic Study*

Multiple tissue blocks from peripheral and central areas of all pulmonary lobes were fixed in Zenker's solution. Alternate serial sections were stained with hematoxylin and eosin, the periodic acid-Schiff, and Verhoeff's elastic tissue methods.

The anatomical divisions of the finer air passages and their component parts were measured by study of serial sections in normal lungs and in those showing vesicular and bronchiolar emphysema. Results of the measurements are summarized in Table II, the mean values being

TABLE II

*Measurements* of Terminal Bronchioles and Elements of the Respiratory Units in Normal Lungs and in Hypertrophic Emphysema*

| Structure | Normal adult lung | Vesicular emphysema | Case 1 Bronchiolar emphysema | | Case 2 Bronchiolar emphysema | |
|---|---|---|---|---|---|---|
| | | | Mild | Advanced | Mild | Advanced |
| **Terminal bronchiole** | | | | | | |
| Diameter of lumen | 0.75 (0.31 –1.20) | 0.18 (0.05 –0.37) | 0.28 (0.04 –0.50) | 0.26 (0.03 –0.54) | 0.22 (0.04 –0.50) | 0.16 (0.05 –0.38) |
| (Distal end with cystic change) | | | 0.58 (0.16 –0.75) | 1.21 (0.56 –1.97) | 0.96 (0.53 –1.66) | 1.03 (0.52 –1.85) |
| Thickness, muscularis | 0.044(0.019–0.085) | 0.043(0.026–0.064) | 0.040(0.028–0.056) | 0.065(0.037–0.106) | 0.034(0.024–0.047) | 0.043(0.029–0.067) |
| (Distal end with cystic change) | | | 0.090(0.074–0.113) | 0.150(0.062–0.431) | 0.074(0.042–0.095) | 0.076(0.030–0.132) |
| **Respiratory bronchiole** | | | | | | |
| First order, length | 1.33 (0.71 –1.79) | 2.17 (0.92 –3.60) | 1.83 (1.23 –3.12) | 3.31 (2.42 –3.83) | 2.91 (2.11 –3.58) | 3.05 (2.63 –3.91) |
| Second order, length | 0.69 (0.57 –0.84) | 0.85 (0.60 –1.04) | 0.82 (0.64 –0.91) | 1.73 (1.17 –2.21) | 1.17 (0.88 –1.46) | 1.42 (1.37 –1.48) |
| Diameter, proximal lumen | 0.44 (0.22 –0.83) | 0.42 (0.14 –0.72) | 0.52 (0.34 –0.78) | 1.20 (0.50 –2.91) | 0.69 (0.33 –1.34) | 1.14 (0.59 –2.33) |
| Diameter, distal lumen | 0.30 (0.17 –0.87) | 0.32 (0.04 –0.54) | 0.27 (0.07 –0.58) | 0.95 (0.20 –1.78) | 0.31 (0.03 –0.81) | 0.86 (0.14 –1.92) |
| Thickness, proximal muscularis | 0.023(0.014–0.031) | 0.027(0.015–0.038) | 0.063(0.029–0.098) | 0.107(0.039–0.222) | 0.043(0.027–0.056) | 0.056(0.021–0.140) |
| Thickness, distal muscularis | 0.026(0.012–0.060) | 0.023(0.016–0.035) | 0.061(0.021–0.119) | 0.095(0.040–0.165) | 0.037(0.029–0.048) | 0.091(0.041–0.176) |
| Thickness, proximal elastica† | 9.1 (7.0 –12.2)μ | 8.2 (5.9 –10.8)μ | 8.0 (6.1 –9.1)μ | Fragmented | 8.1 (7.6 –9.1)μ | Fragmented |
| Thickness, distal elastica† | 14.7 (10.6 –20.4)μ | 14.2 (13.0 –16.4)μ | 14.1 (13.3 –14.9)μ | Fragmented | 14.0 (13.2 –14.8)μ | Fragmented |
| **Bronchiolar alveoli** | | | | | | |
| Diameter | 0.105(0.035–0.215) | 0.147(0.102–0.215) | 0.157(0.078– 0.353) | 0.261(0.020–0.854) | 0.168(0.093–0.350) | 0.208(0.025–0.733) |
| Diameter of pores of Kohn‡ | 3.5 (2.8 –4.8)μ | 4.9 (2.8 –10.6)μ | 14.4 (10.9 –17.9)μ | Obliterated | 12.7 (10.8 –14.1)μ | Obliterated |
| **Alveolar ducts** | | | | | | |
| Length | 1.92 (1.10 –3.50) | 3.28 (1.92 –7.17) | 1.86 (1.12 –3.16) | Obliterated | 1.88 (1.14 –2.93) | 1.65 (1.31 –2.17)¶ |
| Diameter, proximal lumen | 0.27 (0.20 –0.40) | 0.32 (0.13 –0.49) | 0.18 (0.04 –0.33) | Obliterated | 0.16 (0.10 –0.24) | 0.06 (0.02 –0.14) |
| Diameter, distal lumen | 0.15 (0.08 –0.20) | 0.31 (0.24 –0.40) | 0.13 (0.06 –0.27) | Obliterated | 0.13 (0.09 –0.17) | 0.04 (0.03 –0.05) |
| Thickness, proximal muscularis | 0.026(0.021–0.031) | 0.025(0.010–0.035) | 0.040(0.021–0.077) | Obliterated | 0.051(0.026–0.081) | 0.043(0.023–0.063) |
| Thickness, distal muscularis | 0.015(0.009–0.021) | 0.018(0.010–0.028) | 0.028(0.014–0.041) | Obliterated | 0.030(0.024–0.033) | 0.026(0.021–0.031) |
| **Ductal alveoli** | | | | | | |
| Diameter | 0.098(0.067–0.155) | 0.436(0.112–1.501)§ | 0.228(0.116–0.369) | Obliterated | 0.219(0.087–0.358) | 0.565(0.172–1.540)¶ |
| Thickness of septa | 11.6 (8.8 –15.1)μ | 8.1 (5.3 –12.0)§μ | 17.9 (11.6 –27.0)μ | Obliterated | 21.4 (12.1 –34.3)μ | 21.9 (12.2 –47.8)μ |
| Thickness of elastica | 1.41 (0.54– 2.80)μ | 1.41 (0.54 –2.28)μ | 3.91 (1.87 –7.81)μ | Obliterated | 3.48 (2.06 –5.26)μ | 2.57 (1.10 –4.84)μ |
| Diameter of pores of Kohn‡ | 3.8 (2.1 – 5.4)μ | 7.4 (6.3 –9.2)‖μ | 5.5 (1.7 –8.2)¶μ | Obliterated | 2.8 (1.4 –4.2)¶μ | None observed |

* All measurements in millimeters except as indicated.

† Total thickness of elastic fibers located on one side of bronchiolar wall in sections cut at 6μ.

‡ Measured with the alveoli in an incompletely expanded state.

§ Ruptured alveoli and their septa are not included.

‖ Does not include emphysematous fenestrae. Intact pores are not numerous since they often appear to be points of rupture.

¶ Very few of these structures present.

given first, followed by the extreme ranges in parentheses. Such data contributed important information to an understanding of the pathogenesis and nosologic aspects of bronchiolar emphysema.

### *Microscopic Description*

Although there were some minor differences of detail which will be indicated, the microscopic appearances of the lungs in the two cases were similar and will be described together. In general, the most advanced stage of pathologic modification was seen in the basal and marginal regions of the lungs, while a gradual diminution in severity of the changes was observed as the hili and apices were approached. This transition was more evident in the central parts of all lobes and especially throughout the upper lobes in case 1. In case 2 the changes were more diffuse, but the same tendency was noted.

In areas of more advanced pathologic change the lung tissue was composed of dense intermingled masses of fibrous, elastic, and muscular tissue among which were interspersed a large number of irregular cystic cavities of varying size and shape. That these cysts were derived from respiratory bronchioles, rudimentary alveoli, and sometimes in part from the lower ends of terminal bronchioles by confluence with the former was evident from examination of their lining as well as their frequent connection. The largest cysts were formed by both elongation and dilatation of the entire length of respiratory bronchioles of the first order, occasionally with involvement of the lower terminal bronchioles. Somewhat smaller cysts originated in the same manner from respiratory bronchioles of the second order. In many instances the ectasia was more pronounced in the upper or proximal part of both orders of respiratory bronchioles. Except for the cysts partially derived from portions of the terminal bronchioles, nearly all of these cavities were lined partly by ciliated columnar epithelium which gradually underwent a series of transitions to a low cuboidal type with an absence or paucity of goblet cells. The former were lined largely by ciliated columnar epithelium and presented numbers of goblet cells only along their proximal borders. The lining of a few cavities was transformed from its original condition by flattening, apparent loss of cilia, and squamous metaplasia. In rare instances there was no perceptible epithelial investment.

The walls of both the cystic respiratory and lower terminal bronchioles were markedly thickened by fibrosis and hypertrophy of their muscularis (Fig. 2). Their elastica was not decreased, but was fragmented and observed in broken and frayed strands within or immediately beyond the muscularis. The smallest cysts were formed by simple

‡ Measured with the alveolar [illegible]
§ Ruptured alveoli and their septa are not included.
‖ Does not include emphysematous fenestrae. Intact pores are not numerous since they often appear to be points of rupture.
¶ Very few of these alveolar [illegible]

dilatation of rudimentary alveoli. Many were enmeshed within hypertrophied bronchiolar smooth muscle, and others were compressed and deformed by fibrous tissue so that they appeared as atretic luminated structures (Fig. 3). All were noted to be completely lined by columnar or cuboidal epithelium, including the alveolar aggregates (alveolar sacs) which were lined entirely by low cuboidal cells, unlike their appearance in normal lungs where this lining is usually imperfect. The interalveolar pores of all of these alveoli were obliterated by connective tissue so that the small cysts ended as blind cavities except for the communications with their parental respiratory bronchioles. A small circumscribed nidus of neoplastic epithelial cells was found arising from the lining of a cystic respiratory bronchiole in one section from case 1. There was no actual invasion of surrounding tissue, pleomorphism, or evidence of mitotic activity, and the lesion was considered to be a bronchiolar adenoma. Many of the cysts contained epithelial débris and varying numbers of inflammatory cells. This exudate consisted almost entirely of polymorphonuclear leukocytes with a significant absence of alveolar phagocytes. The inflammatory response was more extensive in some areas in case 2.

Between the cystic spaces was a compact stroma composed of dense fascicles of collagen, large numbers of conspicuous elastic fibers in interwoven strands and coils, scattered and often disorganized bands of hypertrophied smooth muscle, thick-walled blood vessels, and deposits of anthracotic pigment. Such materials appeared to represent vestiges of previously functional structures of distal respiratory segments no longer extant. In case 1 the stroma contained no patent alveolar ducts or distal alveoli and the proximal segments of the cribellus ended as blind cysts, while in case 2 the same situation was prevalent but a few enclaves of these structures were discerned. Concerning the latter, the alveolar ducts were short, either narrow or focally dilated, showed muscular hypertrophy, and sometimes exhibited secondary epithelization by small cuboidal cells continuous and apparently identical with those of the lower respiratory bronchioles to which they could often be traced. The distal alveoli were usually very large, formed no elaborate aggregates, and had thick septa with no interalveolar pores. Some were distended and others were so encompassed by connective tissue that they were collapsed and often showed secondary epithelization by lining rims of cuboidal cells. Throughout the stroma in both cases there was an inflammatory infiltration of varying degree composed mostly of lymphocytes, although plasma cells, polymorphonuclear leukocytes, and histiocytes were noted in areas. Entire or portions of terminal bronchioles uninvolved by the cystic

process exhibited luminal narrowing, deformity, and frequently focal lymphocytic collections in the surrounding connective tissue.

Areas of minimal pathologic alteration showed essentially similar changes of less degree. Usually the dilatation of the respiratory bronchioles was either confined to, or most marked in their upper portions. In the latter instance the dilatation extended peripherally to involve most of the course of these structures and occasionally proximally to include the lower terminal bronchioles. Rudimentary alveoli were distended proportionately to the degree of dilatation in the respiratory bronchiole at their respective points of origin, and their interalveolar pores were correspondingly enlarged. These cystic portions of proximal segments of respiratory units did not usually end as blind cavities, but extended into hypoplastic distal segments which were less numerous and elaborate than average. The alveolar ducts were short and narrow and showed smooth muscle hypertrophy, prominent elastica, and deficient alveolization. Sometimes stenosis of the lumina was noted. The distal alveoli were larger than usual, arranged in uncomplicated aggregates, and had thick intact septa containing an abundance of collagenous and elastic fibers and a paucity of somewhat dilated interalveolar pores (Fig. 4). The striking thickness of the alveolar walls was found to be due to both an excess of collagen and conspicuous elastic lamellae. Some scattered alveolar phagocytes were found in the lumina of all parts of the respiratory units, in sharp contrast to their absence in the advanced stage of pathologic change. These modifications were progressively less striking toward the hili and apices, especially in case 1 in which only slight hypoplasia of distal respiratory segments was observed in association with the well known changes of vesicular emphysema.

In summary, the pathologic process exhibited intricate patterns of groups of interconnecting, rounded or asymmetric cysts of varying size. Each group consisted of a dilated proximal respiratory segment comprised of a single respiratory bronchiole and its appendages with hypertrophy of the bronchiolar muscularis, impairment of its elastica, and fibrosis. Many proximal segments ended as blind, multibulbous sacs forming abbreviated respiratory units. These were embedded in a dense fibromyo-elastic matrix composed of stenotic, hypoplastic distal segments or the residual stroma derived by the obliteration of such structures. Such alteration in more advanced stages produced respiratory units which were apparently impervious to the egress or ingress of collateral air and incapable of more than a modicum of requisite hemogaseous exchange.

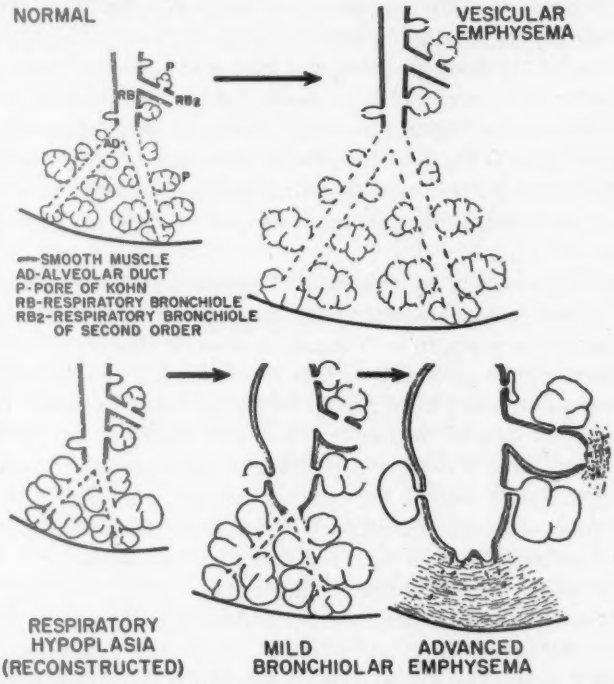
## Discussion

There has been considerable confusion concerning suitable criteria for the classification of emphysema, bronchiectasis, and the so-called congenital and acquired forms of cystic disease of the lungs. It is our opinion that the expression cystic disease of the lungs should be reserved for congenital lesions, and that the term acquired cystic disease should be abandoned. Both consistent analysis and simplicity of classification indicate that all examples of acquired ectasia of the lungs be grouped under one of two distinct clinicopathologic entities, bronchiectasis (including bronchiolectasis), and pulmonary emphysema. Excluding the compensatory and postural types of emphysema, the common, diffuse form of this disease is preferably called hypertrophic emphysema.

*Hypertrophic emphysema* is defined as a diffuse disease of the lungs characterized by an acquired ectasia of the respiratory units which are enlarged both by over-all lengthening and segmental dilatation, associated with focal impairment of their elastica at the site of the dilatation and, as the condition becomes established, with hypertrophy of smooth muscle elements. Two varieties of hypertrophic emphysema are recognized. When the focal dilatation and elastic tissue damage occur in the proximal or bronchiolar segment of the cribellus, as in the cases presented, the disease is called *bronchiolar emphysema*. When the distribution of these changes is found in the distal or ductal segment, the term *vesicular emphysema* is appropriate. This difference is readily appreciated by examination of Figures 5 to 7. Both types appear to have the same etiology and several similar morphologic and clinical features, but differ in pathogenesis.

The etiology of hypertrophic emphysema has never been fully explained. It must be noted that the lungs of mammals, including man, are predisposed to the development of emphysema as a result of the unfavorable morphologic evolution of a two-way air-conducting system which, when partly obstructed, permits more complete inspiration than exhalation. This is in contrast to the physiologically more efficient anatomical specialization of recurrent bronchi and pneumatic bones found in certain species of birds. In man, hypertrophic emphysema has been attributed to a variety of obstructive disturbances occurring in the immediate prerespiratory terminal bronchioles. The various theories include a check-valve bronchiolar obstruction, spasm of the muscularis, and chronic stenosing bronchiolitis, all associated with chronic strenuous coughing. Without discussing the merits of each, pathophysiologic considerations indicate that a precipitating mechan-

ism is common to all. This is aerodynamic trauma leading to the destruction of elastic tissue within the respiratory units at its weakest point. For over a century some writers (among them Calma,[9] Pepere,[10] and Dey[28]), without offering any evidence, have indicated a congenital or dispositional defect of elastic tissue as a cause of emphysema, while others[29-32] have denied or failed to corroborate this. We find no reason to support the former assumption, and a comparison of the measurements of the elastica of normal and emphysematous lungs offers no confirmation (Table II).




Text-figure 1. Schema of changes in respiratory unit of normal lungs and those with respiratory hypoplasia leading to development of vesicular and bronchiolar emphysema.

The complex pathogenesis of bronchiolar emphysema can be explained as a concurrent interplay of three separate predisposing factors, all of which must be present. They are hypoplasia of the distal segment of the respiratory unit, an inherent structural weakness of the myo-elastic wall of the upper respiratory bronchiole, and the pathophysiologic components which enhance this weakness. The first is unique and is the crucial factor which predetermines that the lesions of bronchiolar emphysema will develop instead of those of vesicular emphysema. The second factor is a constant and is found in all normal

lungs, and the third is found in all examples of hypertrophic emphysema. The development of bronchiolar emphysema as contrasted to that of vesicular emphysema is schematically depicted in Text-figure 1.

### *Factor of Respiratory Hypoplasia*

Variations of lung structure may assume several forms. *Respiratory hypoplasia* is the term we have adopted for a condition in which there is less complete differentiation of the respiratory units in their distal segments, so that relatively fewer, smaller, and less extended alveolar ducts give rise to a limited number of comparatively large peripheral alveoli which are arranged singly or in less elaborate aggregates than in the more fully developed lung and have walls of more than standard thickness with a paucity of interalveolar pores. The pronounced thickness of the alveolar septa is accompanied by an abundance of collagen and elastic fibers. On the other hand, *pulmonary hypoplasia* must be distinguished from this condition, and defines situations in which one or more bronchopulmonary segments are lacking or smaller than average.

No previous study of respiratory hypoplasia seems to have been made, although MacMahon[33] has described an apparently identical condition in the newborn as "congenital alveolar dysplasia." In severe cases insufficient respiratory surface to sustain life resulted and death occurred a few hours after birth. In milder cases he found that the alveolar "retardation" was compatible with viability, but he did not determine if there was a predisposition to subsequent pulmonary disease. MacMahon was of the opinion that the condition was a congenital anomaly of obscure etiology having its origin in early embryonal life, and noted that it had been found in identical twins.

Embryologic considerations indicate that respiratory hypoplasia may result from an inequality of the entodermal epithelium of bronchial buds and the mesenchyme into which it invaginates. This disproportion is reflected in the anatomical construction of the distal segments of the cribellus as hypoplasia. It would appear that the preponderance of mesenchyme is produced by a deficiency of entoderm, not an excess of mesoderm. Support for this hypothesis is provided by the more pronounced basal and marginal distribution of hypoplastic respiratory units, for it is in these regions furthest removed from the central and cephalad position of the primary tracheal entodermal bud that one would expect the entodermal component to show the poorest development. It is of interest that respiratory hypoplasia is a morphologic variation bearing strong analogies to a more primitive type of mam-

malian lung in which the proximal segment of the respiratory unit or archicribellus is prominent and the more specialized distal segment or neocribellus is less developed.[34]

When hypertrophic emphysema develops in lungs showing respiratory hypoplasia, it will be invariably of the bronchiolar type due to the greater thickness of the walls of the structures of the distal segments of the cribellus and especially the greater strength of the alveolar septa. This is demonstrated by the microscopic features of the lesions and the measurements of the anatomical elements of the distal respiratory segments (Table II) in these two cases of bronchiolar emphysema. The marked thickness of the septa and elastic tissue of the distal alveoli manifestly provided twice or more the margin of safety to increased aerodynamic pressure than normal alveoli. In bronchiolar emphysema the distal alveoli, apparently originally larger than average, and their very limited number of small interalveolar pores underwent some degree of dilatation in accordance with the general changes of hypertrophic emphysema. The dilatation of these structures, however, was much less striking than in vesicular emphysema and the thinning or rupture of the septa with elastic impairment was absent.

### *Myo-elastic Factor*

The myo-elastic elements of the respiratory unit, including the elastic fibers of all its alveoli, form a continuous and single morphologic and physiologic system. The weakest part of the proximal segment of the cribellus is found in the wall of the upper respiratory bronchiole. At this point support is provided only by thin, oblique and discontinuous bundles of smooth muscle and delicate longitudinal elastic fibers which provide a small margin of safety to aerodynamic pressure. The muscularis of the proximal respiratory bronchiole is normally slightly thinner than that of the lower part of this structure or of the beginning portions of the alveolar ducts as is shown by our measurements (Table II). Furthermore, the upper respiratory bronchiole has a muscularis which does not entirely envelop its circumference and is partly absent on the side which is free of rudimentary alveoli and lies near the course of the accompanying branch of the pulmonary artery. Such lacunae in the muscular net are filled with fine fibrous and elastic connective tissue. All of the above findings agree with the meticulous observations of Baltisberger.[35] Related information is supplied by Orsós[31] who determined that the elastica of the terminal part of the respiratory bronchiole and beginning portion of the alveolar ducts forms a strong net, and also has some circular fibers tending to constitute a ring.

*Pathophysiologic Factor*

From our observations the normal physiologic mechanisms of respiration as described by Macklin[36] and Bayliss and Robertson[37] are accentuated and modified in hypertrophic emphysema. In this disease the terminal bronchioles, altered by a chronic stenosing bronchiolitis and fibrosis, become less capable of undergoing their share of dilatation and elongation during inspiration, so that even greater movements are required of the more distal tubular structures of the respiratory units. In addition, the presence of an obstructive disturbance in the terminal bronchioles greatly increases the intracribellar air pressure and the respiratory units suffer a chronic hyper-inflation. As a result of these two occurrences, the respiratory bronchioles and alveolar ducts are in a perpetual state of attenuation and distention even at the end of expiration, and during inhalation these changes are augmented proportionately. When further inspiration follows, their smooth muscle bundles become less capable of opposing dilatation and their elastica is stretched primarily by hyper-attenuation. Thus, a markedly reduced margin of safety to the dilating effect of increased air pressure is produced at the weakest point in the tubular myo-elastic system, and this weakness becomes most manifest when these structures are situated in the basal and marginal portions of the lungs due to the greater excursion and elongation in these regions.

In areas of mild involvement in the cases studied there was little or no fibrosis or hypertrophy of smooth muscle elements. The reports of Pepere,[10] Loeschcke,[11] and Romhányi and Maccone[12] in young individuals are apparently examples of acute bronchiolar emphysema in this state of development. However, in most cases, including those reported here, the disease pursued a course of long duration. Hypertrophy of the smooth muscle of the respiratory units apparently resulted from a gradual compensatory response primarily to dilatation, and in only a slight degree to hyper-attenuation, as is shown by contrasting increases of muscularis in vesicular and bronchiolar emphysema (Table II). Since the dilatation was focal, it produced a pronounced hypertrophy of the muscularis of the proximal respiratory bronchioles and of contiguous portions of the lower terminal bronchioles. On the other hand, slight hypertrophy of the smooth muscle of more or less all of the cribellus was noted in both types of chronic hypertrophic emphysema. In the basal and marginal regions of the lungs where the respiratory units were subject to the greatest lengthening and therefore sustained the greatest ectasia, the muscular hyper-

trophy and elastic tissue damage became most prominent and extended peripherally. Consequently, in these areas the muscularis of the lower respiratory bronchioles and beginning portions of the alveolar ducts, normally the thickest smooth muscle found in the respiratory unit, underwent hypertrophy which in some cases equalled or exceeded that of the upper respiratory bronchioles. Furthermore, the lumina of the proximal parts of the alveolar ducts, originally narrower than those of the fully developed lung because of the hypoplasia, underwent stenosis as a result of the muscular increase and the associated space-taking effect of the expanding cysts. Superimposed inflammation occurred and fibrosis took place, so that stenosis was followed eventually by obliteration of the distal segments. A vicious circle was established in which there was increasing fibrosis of entire respiratory units with replacement of their distal segments by more and more collagenous tissue as a reparative response to chronic inflammation. Eventually the bronchiolar muscularis and fibrous tissue of the stroma became so increased that some of the previously distended proximal alveoli were compressed and obliterated.

The final lesion is comparable to the morphologic pattern of a primitive type of vertebrate lung which presents only an epithelium-lined archicribellus and lacks the differentiation of anepithelial alveolar ducts and distal alveoli as separate and specialized structures. Insufficient and unsatisfactory respiratory surfaces result, and fewer and fewer functioning alveoli remain until embarrassed hemorespiratory exchange and cor pulmonale terminate the life of the patient.

The acquired and progressive nature of bronchiolar emphysema is confirmed by the long clinical histories of the patients and roentgenographically by the advance of the lesions in the chest films over a period of more than 5 years in our first case and during an interval of 7 years in the first case of von Stössel.[8]

Patients with bronchiolar emphysema show essentially the same physical and laboratory findings as do those with vesicular emphysema. Their common clinicopathologic features support the conclusion that they are varieties of the same disease and should be classified as types of hypertrophic emphysema. At least four of the twelve recorded cases, including our first case, showed the lesions of bronchiolar emphysema combined with those of vesicular emphysema, further substantiating this point. In bronchiolar emphysema there is dyspnea, inability to hyperventilate on exercise, impaired elastic recoil, inability to complete pulmonary systole, embarrassed hemorespiratory exchange, and cyanosis. Associated are a barrel-shaped chest, cor pulmonale, a

subclinical "respiratory" acidosis, and occasionally spontaneous pneumothorax and secondary polycythemia.

This disease would seem to be more common in males, but in view of the limitation of this series to twelve cases and the rejection of the questionable examples of "cystic disease" and "myomatosis," all of which occurred in females, any conclusion purporting to establish a predominance in males seems unjustified. While bronchiolar emphysema is the less common type of hypertrophic emphysema, we do not believe it is as rare as the number of cases cited would indicate. Undoubtedly, examples have masqueraded under the all-inclusive and deceptive terminology of congenital cystic disease of the lungs, or if they were recognized correctly as acquired lesions, as honeycomb lungs or myomatosis.

Muscular hypertrophy and fibrosis are outstanding features of chronic bronchiolar emphysema. Consequently, most observers have been led to emphasize these characteristics and have used such purely descriptive terms as muscular cirrhosis and cystic cirrhosis to describe the disease, while its fundamental emphysematous nature has been obscured. These terminologies are also objectionable because other pulmonary diseases, such as acquired cystic bronchiolectasis (honeycomb lungs),[38,39] and congenital pulmonary cystic lymphangiectasis[40] may give the lungs a grossly "cirrhotic" appearance. Great care must be taken to distinguish cases of cystic bronchiolectasis with muscular hypertrophy, which at times may be associated with a secondary hypertrophic emphysema, from true bronchiolar emphysema. Primary bronchiolectasis is characterized by dilatation of entire terminal bronchioles and often parts of the preterminal bronchioles with cartilage plates, and apparently results in the formation of somewhat larger cysts.

## Summary

The morphologic and clinical features of two examples of emphysema of an unusual type are presented. Review of the world literature revealed only ten cases sufficiently documented to be considered examples of this lesion, which is more aptly designated as bronchiolar emphysema rather than cystic or muscular "cirrhosis" of the lungs. The former correctly relates this process to emphysema and identifies the principal site of morphologic alteration, whereas the latter term refers only to the gross appearance of the involved lungs in an advanced stage of the disease.

The complex pathogenesis of bronchiolar emphysema is attributed to respiratory hypoplasia, an uncommon and atavistic variation of nor-

mal lung structure, and to certain anatomical and pathophysiologic factors leading to the formation of emphysematous cysts derived from the respiratory bronchioles and their appendages. The presence of respiratory hypoplasia appears essential for the development of bronchiolar rather than vesicular emphysema.

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[ *Illustrations follow* ]

## LEGENDS FOR FIGURES

FIG. 1. Case 1. "Cirrhotic" appearance of external surface of lung.

FIG. 2. Case 1. Cystic respiratory bronchiole, revealing marked hypertrophy of muscularis, fragmentation of elastica, and luminal exudate. Verhoeff-van Gieson's stain. × 140.

# MAMMOTH

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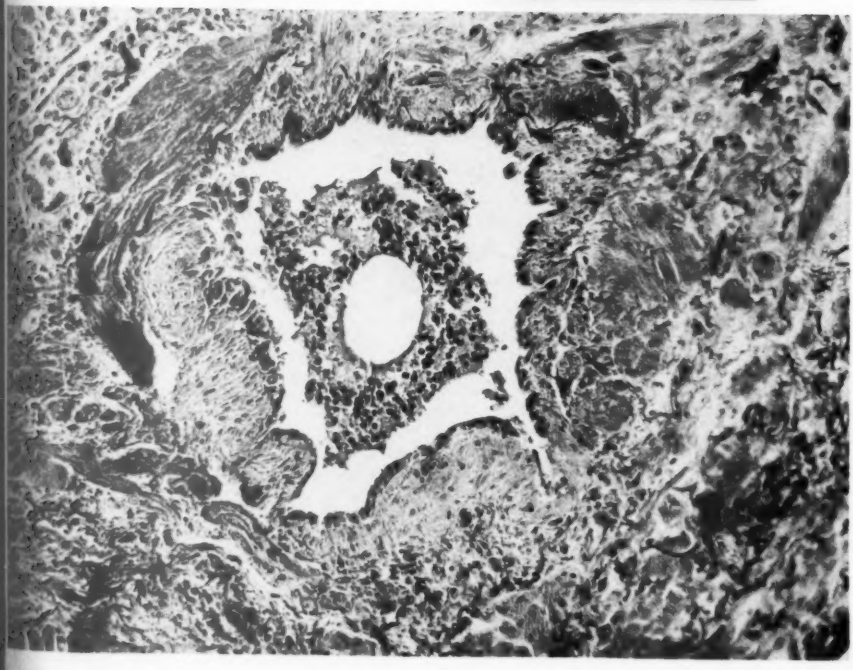

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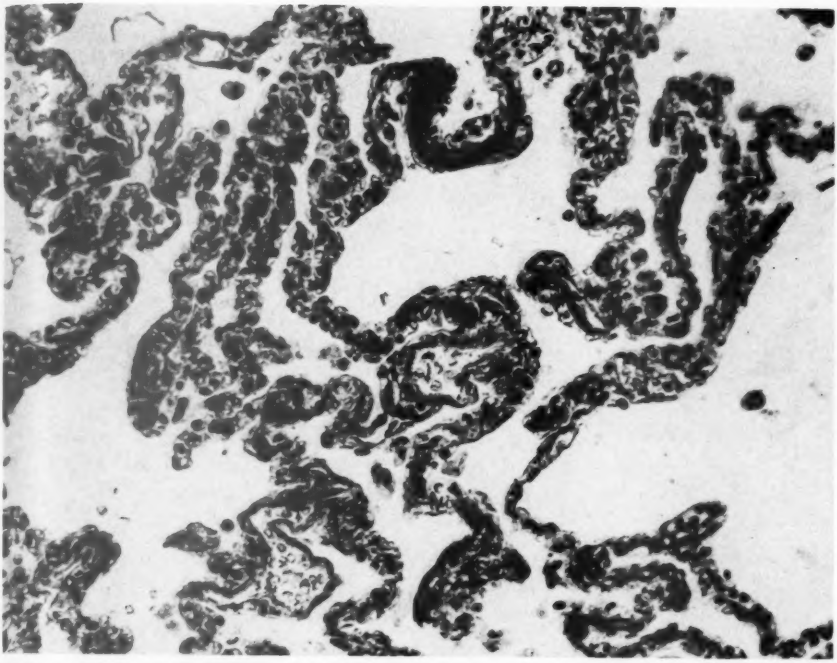
FIG. 3. Case 2. Tangential section of two deformed respiratory bronchioles arising from a terminal bronchiole. Cystic, rudimentary alveoli surrounded by dense stroma with anthracotic pigment. Hematoxylin and eosin stain. × 35.

FIG. 4. Respiratory hypoplasia evidenced by small numbers of large alveoli with thick septa containing abundant elastic fibers. Verhoeff-van Gieson's stain. × 200.

FIGS. 5, 6, and 7. Low-power comparison of sections of lung from normal tissue (Fig. 5), vesicular emphysema (Fig. 6), and bronchiolar emphysema (Fig. 7). Verhoeff-van Gieson's stain. × 15.



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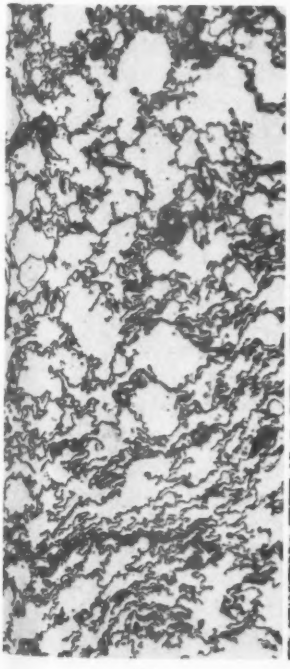

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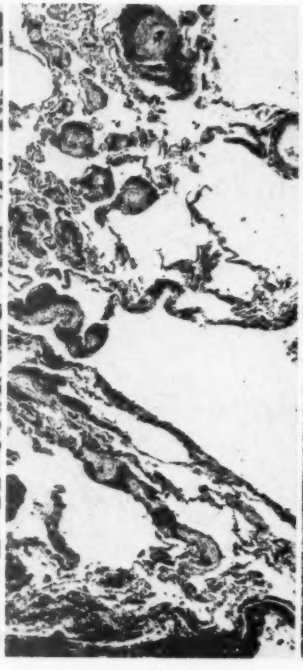

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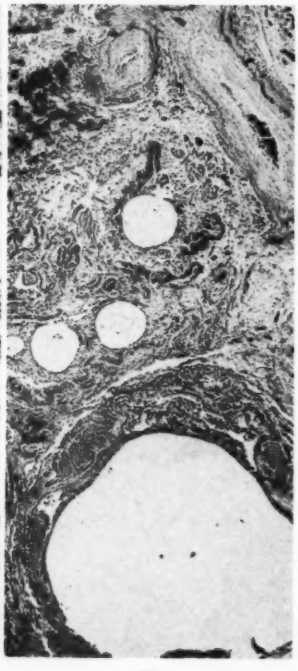

7

# MYCOBACTERIUM ULCERANS

## Pathogenesis of Infection in Mice, Including Determinations of Dermal Temperatures *

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One of the most notable characteristics of *Mycobacterium ulcerans* is the temperature requirement for its multiplication *in vitro* and *in vivo*.[1] In artificial mediums, growth occurs at 33° C., not at 37° C. Clinically, lesions occur usually in the integument. In the experimental animal also, the integument is affected, and lesions may occur in organs near the surface of the body, such as the testis and epididymis. Predilection for cutaneous tissues is shared also by *Mycobacterium leprae* and *Mycobacterium balnei*[2,3] and by the mycobacteria presumably responsible for the disease known as lepra bubalorum, which occurs in the water buffalo in Celebes.[4] So-called skin tuberculosis of cattle is another condition related to an undetermined form of mycobacteria, which, like the examples previously mentioned, occurs in the skin or the subcutaneous tissue.[5]

Considerable information concerning the pathogenicity of *Myco. ulcerans* for experimentally infected animals was recorded in the first account of the organism by MacCallum, Tolhurst, Buckle, and Sissons.[1] Of the animals tested, rats and mice had the greatest susceptibility. In rabbits lesions limited to the epididymis occurred. Attempts to infect lizards and one chicken were unsuccessful.

The most extensive observations on the pathogenic behavior of *Myco. ulcerans* in mice are those published recently by Fenner.[6] From the results of comprehensive studies, Fenner concluded that in man and in experimental animals the pathogenic behavior and growth of *Myco. ulcerans* (and *Myco. balnei*) are governed "upon a narrow temperature range."

In a previous report[7] the results of a study to determine the response of infections by *Myco. ulcerans* in mice to various antimicrobial agents were recorded. The lesions that developed subsequent to intravenous inoculation in the tail occurred predominantly in the skin and other soft tissues of this appendage, including the bone marrow. To obtain further information regarding a possible correlation of route

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† The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.

of inoculation with the distribution of lesions in the infected animal, another group of experiments was done. In addition, it seemed desirable to obtain some definitive information concerning the temperature of the skin of tails of normal mice. For comparison, the temperature of the interior of the body as measured in the rectum was determined also.

For convenience, an account of the observations which follow is presented under two headings: Part 1: Pathogenesis; Part 2: Temperature Determinations in Normal Mice.

## Part 1: Pathogenesis

### *Methods*

Forty white mice, strain C, were divided into four groups of ten animals each. Each animal was inoculated with a suspension of a 20-day-old culture of *Myco. ulcerans*.* The suspension had a density equal to tube 0.5 of the McFarland nephelometer. The route of inoculation, which was different for the respective groups, and the amount of inoculum for each group were as follows: intravenously via tail vein, 0.1 ml.; into subcutaneous tissues of tail, 0.05 ml.; into the periosteum of the left femur, 0.1 ml.; and intracerebrally, 0.05 ml.

### *Mortality and Survival Time*

The experiment was terminated 120 days after the animals had been inoculated. Of the 40 mice comprising the four groups of 10 each, only 12 were living at the 120th day. The others of the original total of 40 had died before the termination of the experiment. Owing to postmortem changes, not all were suitable for subsequent study. The number of animals in the respective groups from which material was available for microscopic study was as follows: inoculated intravenously, nine; inoculated into the subcutaneous tissue of the tail, six; inoculated over or into the periosteum of the femur, ten; inoculated intracerebrally, three. One animal died after 4 days and was excluded.

Material examined microscopically from the mice in the respective groups included tissue in most instances from tail, femorotibial joint and adjacent muscles, liver, testes, spleen, kidneys, lungs and, for those animals inoculated intracerebrally, the cerebrum. Duplicate sections were made of all tissues examined. One section was stained with hematoxylin and eosin. To reveal acid-fast microorganisms if present, one section was stained with carbolfuchsin-hematoxylin.

Data on the distribution of lesions and the structural changes observed in the mice in the respective groups follow.

* Harvey strain obtained in 1950 through the courtesy of Mr. Glen Buckle, Bacteriology Department, Alfred Hospital, Prahran, S. 1, Victoria, Australia.

*Group Inoculated Intravenously via Tail Vein*

With one exception to be noted later, the lesions associated with demonstrable acid-fast bacilli were confined to the various structures of the tail. Characteristic lesions were present in each of the tails available for microscopic examination. The histopathologic features of the tissues of the tails of mice inoculated with *Myco. ulcerans* have been described previously.[7] However, in the material examined, the severity of the disease varied and did not seem to be correlated with the duration of the infection. The shortest period after inoculation of the material studied was 44 days and the longest period was 120 days. No acid-fast organisms or other signs of infection were observed in the myeloid tissue of four of the tails. In two animals a few to many acid-fast bacilli occurred in several lacunae of the caudal vertebrae.

In this group no evidence of infection by *Myco. ulcerans* was found in any of the other organs except in one lung of a mouse that died 75 days after receiving the infectious inoculum. In this instance, a considerable portion of one lobe was solidified. The most striking feature of the pulmonary lesion was a dense nodular condensation of acid-fast bacilli (Fig. 1). The organisms were present in tremendous numbers, and it was impossible to ascertain with certainty whether the bacteria occupied intracellular positions. In fact, the density of the organisms in the lesion was such as to make it difficult to recognize tissue cells of any kind except near the periphery. Although most of the bacteria were within a single focus, a few organisms were noted in the surrounding exudate. Another smaller focus of acid-fast bacilli was noted in another lobe of the same lung.

*Group Inoculated Into the Subcutaneous Tissue of the Tail*

Information regarding external lesions of infection with *Myco. ulcerans* was available for only six of the ten mice inoculated. The tail of each of the six mice showed gross evidence of infection, and this was confirmed microscopically. The lesions were confined largely to the integument, with extension to the area of the caudal muscles and tendons. In none of the six animals in this group was the infectious process seen in the myeloid tissues. Likewise, no signs of infection with *Myco. ulcerans* were found in any of the other situations examined. These included femorotibial joint and associated soft tissues, testes, lungs, liver, spleen, kidneys, and cerebrum.

*Group Inoculated Over or Into the Periosteum of the Femur*

Tissues for microscopic examination were available from each of the ten animals in this group. The first animal died 18 days after inoculation and four were sacrificed for necropsy after 120 days. At necropsy,

approximately 10 weeks after inoculation, a considerable portion of the tail of one animal was missing. Microscopically, in the remaining portion of the tail there was considerable involvement of the myeloid tissues, with several dense foci of acid-fast bacilli. No demonstrable lesions existed in the integument or the underlying zone of caudal muscles and tendons. In one other mouse of this group that died after 69 days, there was a minimal amount of involvement of the myeloid space of the tail. This was recognized by the presence of a large number of acid-fast bacillary cells. No lesions were noted in the tails of the eight other mice in this group.

Since it was presumed that the animals in this group had all been inoculated into or upon the periosteum of the distal end of the femur, tissues from the femorotibial articulation of these mice were examined with particular interest. In three animals, which died at 67, 69, and 88 days, respectively, after inoculation, severe myositis with marked edema and cellular infiltration of the dermis was observed in the soft tissues surrounding the femorotibial joint. In one animal, no acid-fast bacilli were found in the process involving the muscles, although a few organisms were present in the inflammatory exudate of the dermis.

In the animal that died 67 days after inoculation the muscles some distance from and continuing to their insertion with the epiphyses of the femur and tibia were involved in a severe inflammatory reaction. The most striking feature was an extensive and severe myositis (Fig. 2). This was characterized by retrogressive changes of the muscle fibers, considerable edema, and the presence, within the damaged fibers and in the areas of definite necrosis, of monocytes, acidophilic granulocytes, plasma cells, and a few lymphocytes. The inflammatory process had advanced into the integument with severe edema extending to the epidermal layer. Of much significance was the presence within the muscle of condensations of mycobacteria (Fig. 3). These occurred in foci associated with clusters of phagocytes, which were packed with the acid-fast bacilli. The bacterial accumulations showed an initial predilection for the connective tissue elements between the muscle fibers, and in one area there occurred an elongated collection of the bacteria which extended for some distance under the muscle capsule or epimysium. The bony structures of the epiphyses were not invaded by the infectious agents, and the marrow was apparently not affected.

Myositis occurred also in the animal that died 69 days after inoculation. The structural changes were essentially the same as those described in the preceding paragraph. Although mycobacteria were not observed in the myeloid tissue of those portions of the femur and tibia examined, it is of interest to note that the myeloid space of the tail of

this animal contained a focus of infection in which many acid-fast bacilli were present.

The testes and epididymides from each of the ten mice and the liver, spleen, lungs, and cerebrum from seven were examined microscopically. In none of these tissues was mycobacterial infection observed.

### *Group Inoculated Intracerebrally*

The amount of material available from this group for microscopic study was regrettably small. Owing to postmortem changes, tissues of seven animals were not suitable for subsequent study. Consequently, the descriptive material that follows pertains to only three mice, all of which were sacrificed 120 days subsequent to inoculation.

The cerebrum of the first mouse was devoid of microscopic lesions. Unfortunately, only a small portion of the brain substance was available for study. That this mouse had definitely been infected was evident from the fact that approximately two thirds of the tail had sloughed. More significant, however, was the presence of dense accumulations of acid-fast bacilli among the caudal muscles, tendons, and nerves. Also present among the cells of the myeloid tissue were many similar bacteria occurring singly and in small clumps. Of more than ordinary interest was the presence of a few acid-fast bacilli within the tendons and several of the large caudal nerves. These occurred as single bacteria. The dermis was affected only slightly, the infection being confined largely to the interstitial areas of the caudal muscles and tendons and to the myeloid space. No signs of infection were found in the testes, liver, spleen, or lungs of this animal.

Sections of the cerebrum of the second of the three animals failed to reveal any structural alterations. However, at one margin of the cerebral tissue several acid-fast bacilli, singly and in small groups, were seen. Most of the bacteria appeared to occupy an intracellular position.

As was true of the first mouse of this group, the tail of the second animal was also involved. The soft tissues of the distal 15 mm. of the appendage had disappeared, leaving only the bony structure. Although the epidermis remained intact, there were inflammatory changes in the deeper portion of the corium. These were characterized by edema, congestion, and thrombosis of the blood vessels and by the presence of large numbers of monocytes and some plasma cells. Most of the caudal muscles had undergone retrogressive changes with extensive necrosis. Similar changes of somewhat less severity had also occurred in the tendons. Large numbers of acid-fast bacilli, mostly in dense accumulations or in small clumps, were a striking feature of the infection in-

volving the muscles and tendons. The organisms were especially numerous in the areas between the periosteum of the vertebrae and the overlying soft tissues. A few acid-fast bacilli were noted in the substance of several of the caudal nerves. Except for groups of acid-fast organisms in a few of the lacunae, the osseous tissues of the vertebrae were not affected. Cells of the myeloid series, however, were no longer recognizable and it appeared as though the myeloid tissue had undergone necrosis. Dense clusters of acid-fast bacilli occurred throughout much of the remnants of the marrow, but no cellular response on the part of inflammatory or other cells was observed. No signs of infection with *Myco. ulcerans* were found in the testes, liver, lungs, spleen, or kidneys of this animal.

The third mouse of the group inoculated intracerebrally yielded impressive results indicative of the dissemination of the infection from initial foci in the brain to the caudal appendage. Although the tail was involved severely, evidence of infection of other organs, such as the lungs, liver, spleen, and testes, was not observed.

In this animal the infection in the brain occurred in both hemispheres of the cerebrum and was confined largely to a relatively large focus in each. One of the foci measured 2 mm. and the other 3 mm. at their greatest dimension. The respective lesions were elongated in contour and, in the material stained by hematoxylin and eosin, were characterized by a central area of amorphous material surrounded by a zone of calcium salts (Fig. 4). Peripheral to the zone of calcification were variable numbers of monocytes and plasma cells and an occasional lymphocyte. Giant cells were not seen. The central portion of the lesions contained tremendous numbers of acid-fast bacilli arranged in closely packed, irregular bundles or clumps. In some areas circumscribed by the mineral salts, the bacteria were less numerous and appeared singly or in small groups of a few organisms. Similar organisms also occurred among the calcified material and for a short distance beyond in the deranged glial tissue. Meningitis was not observed.

At necropsy three fourths of the tail of this animal was missing. Microscopically, no abrasions were seen in the epidermis. There was, however, a moderate amount of edema of the subcutaneous tissues, but no acid-fast bacilli were found in the exudate. The caudal muscles, tendons, and nerves were essentially normal in appearance, although in one instance a few acid-fast bacilli were noted in the loose connective tissue peripheral to the epineurium.

The infection in the tail was confined largely to the myeloid tissues. In only a few of the marrow spaces were cells of a myeloid character recognizable. In the remainder of the spaces the cellular contents

were of uncertain character, with cellular outlines barely discernible and nuclei, except for a few shadowy forms, absent. In these areas acid-fast bacilli were present in large numbers (Fig. 5). Most of the organisms were in dense clumps or clusters interspersed by clear spaces, the nature of which was uncertain.

### *Comment*

In the four groups of mice inoculated, the most vulnerable site for infection was the tail, regardless of the site of inoculation. Infection of the bone marrow of the caudal vertebrae seemed dependent on at least a transitory bacteremia from bacteria deposited directly into the blood stream at the time of inoculation or from bacterial foci in the cerebrum or in tissues overlying the periosteum. In the mice inoculated into the subcutaneous tissue of the tail no acid-fast bacillary cells were found in the myeloid elements despite the fact that the inoculation resulted in an infectious process that not only invaded the integument but had advanced inward between the muscles and tendons to the periosteum of the caudal vertebrae.

It may be assumed that, regardless of the site of inoculation and even when a hypodermic needle of the smallest gauge is used, the possibility of inadvertently rupturing or entering a small vessel exists. On the one hand, should this occur, the number of bacteria from the inoculum actually entering the blood stream at a single episode would be relatively small, and these bacteria could conceivably be removed from the circulation by phagocytic cells. On the other hand, if a focus of multiplying bacteria is established in an area distant from the caudal appendage, the subsequent inflammatory changes and destruction of tissue would provide a propitious situation for the entrance of bacteria into the blood stream. Once in the circulation and if in sufficient numbers to avoid complete phagocytosis, metastatic foci could occur where the temperature is favorable. Parenthetically, the observations of Fenner[6] are of interest. According to this author, the inoculation of *Myco. ulcerans* into the footpad of mice is followed by a severe, progressive, local infection with the involvement in many instances of the inguinal lymph node. Visible bacilli can be observed in the liver, spleen, and lungs; yet demonstrable lesions are seldom produced in situations distant from the site of inoculation.

The results following the inoculation of *Myco. ulcerans* into the tail vein failed to indicate any significant correlation between the extent and severity of the process in the caudal appendage and the duration of life after the mice were inoculated. Since the amount of the infective inoculum for each animal was the same, variation in the severity of the

subsequent tissue reactions suggests differences in the native resistance or susceptibility of individual mice to this infection.

The involvement of the marrow of the tail was not observed in the mice that had received the inoculum subcutaneously in the tail. Although the threshold was suitable for the multiplication of the organisms with a marked expression of pathogenicity in the contiguous tissues, the infection did not, in the animals examined, extend into the substance of the caudal vertebrae or to the bone marrow. Perhaps, if the infection had been of longer duration, these last-mentioned tissues, as well as others, would have been affected. In this connection it is of interest to note that in the bone marrow of patients with lepromatous leprosy, lesions associated with the presence of *Myco. leprae* occur in a variable percentage of cases.[8,9]

In the material obtained from the present study and in the material reported on previously,[7] acid-fast bacilli were observed in the peripheral nerves of the tail in a few of the mice. What the true significance of these findings may be is uncertain. The possible rôle of peripheral nerves in the early phases of the pathogenesis of human leprosy has been emphasized in recent years by Khanolkar,[10] who stated: "All leprosy is neural in its inception, in as much as the spread of microorganisms is either in or alor[illegible]nerve fibers in the initial stages." This point of view has not been gen[illegible]cepted.[11] Whether any significance can be attributed to the per[illegible]ves in the natural stages of the pathogenesis of infections with *Myco. ulcerans* is doubtful, if not unlikely. Information on the histopathology of the initial changes which occur in *Myco. ulcerans* infections in man is not known. However, in the original report[1] on *Myco. ulcerans* infection in man it was stated: "No involvement of nerve trunks was observed."

Despite the finding of acid-fast bacilli within the peripheral nerves of mice infected experimentally with *Myco. ulcerans,* such observations were exceptional. Although nerves are numerous among the caudal muscles and tendons, only occasionally were acid-fast bacillary forms detected, regardless of the fact that the extramyeloid infection in the tail was characterized by the presence of many acid-fast organisms in the connective tissues around the muscle tendons and nerves. Frequently, such bacterial concentrations were strikingly evident in the epineurium. Involvement of the perineurium also occurred, but mass invasion of the nerve proper was not observed. When bacteria were found, they were usually within the myelin sheaths, although in a few instances the organisms were apparently within the nerve fibers. Structural changes associated with the presence of the bacilli within the nerves were not noted.

From the limited evidence available, it appears that the constituents of the peripheral nerves form a rather effective barrier to invasion by *Myco. ulcerans*. In the material studied there was no evidence that the peripheral nerves of the tail had any rôle in the pathogenesis of infections with *Myco. ulcerans*. It appears that the invasion of nerves represents a direct extension of the infection previously established from foci in the contiguous tissues. However, the course of events following experimental infections in animals does not necessarily imply that a similar situation obtains following natural infections in man. More precise information regarding this question is desirable.

## Part 2: Temperature Determinations in Normal Mice

The purpose of this experiment was to obtain objective data on the temperature gradient along the tail of the mouse and also to determine the deviation of these temperatures from the general body temperature. The rectal temperature was considered to be representative of the general body temperature. As mentioned previously, stimulus for this study developed from the observation that the tail of the mouse appeared to offer a favorable environment for the multiplication of *Myco. ulcerans*.

### *Method*

Thermistor-thermometers were employed exclusively in this study. Two types of thermistor mountings were employed. The needle (23-gauge) type was preferred for determining temperatures in the tail. The plastic-tube mounting was used for determining rectal temperature.

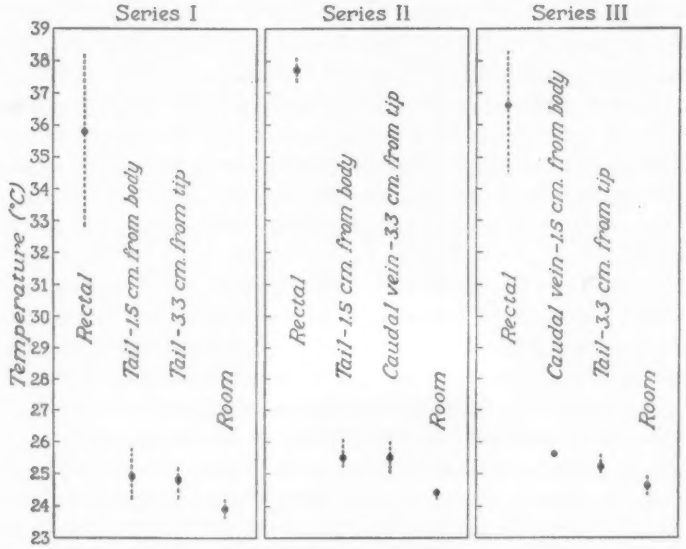
Temperature observations were made on three separate series of normal mice obtained from the same stock as those used in the inoculation experiments. In the first series, subcutaneous temperatures in the tail were recorded at two sites, namely, at about 1.5 cm. from the body and also at approximately 3.3 cm. from the tip of the tail. In the second series, one thermistor was inserted into the caudal vein at the distal end of the tail, the other thermistor being placed in the subcutaneous tissue at the proximal site. In the third series, the proximal thermistor was inserted into the caudal vein and the distal thermistor was placed in the subcutaneous tissue. In each series, rectal temperatures were observed simultaneously.

The mice were comfortably immobilized in a case designed especially for these studies (Fig. 6).

### *Results*

In the first series, of 20 mice, the average rectal temperature was 35.8° C. The average temperature of the tail at about 1.5 cm. from the body was 24.9° C. and the average temperature near the distal end of

the tail was 24.8° C. In the second series, of five mice, the average rectal temperature was 37.7° C. The average temperature of the subcutaneous tissue at the proximal end of the tail was 25.5° C. and the average temperature of the blood within the caudal vein at the distal end of the tail was also 25.5° C. In the third series, of five mice, the average rectal temperature was 36.6° C. The average temperature of the blood within the caudal vein at the proximal end of the tail was 25.6° C. and the average temperature of the subcutaneous tissue at the distal end of the tail was 25.2° C. Text-figure 1 shows graphically a summation of these data.




**Text-figure 1. Average temperatures (dot) and ranges of variation (broken line) recorded for the three series of normal mice.**

## *Comment*

If, in the inoculation experiments previously described, other factors controlling the growth of *Myco. ulcerans* were fairly constant, it is a reasonable assumption that the markedly lower temperature of the tail was a significant factor in accounting for the susceptibility of this structure to the action of this organism. The tail of the mouse is approximately only 1° C. warmer than the room temperature under the conditions of our experiments. On the other hand, the temperature of the tail is approximately 11° C. (almost 20° F.) lower than the rectal temperature of the mouse.

In future explorations of the relation of the temperature *in vivo* to the multiplication of *Myco. ulcerans,* an attempt should be made to determine whether or not the temperature of the tissues constituting the reaction of the body to the infection is the same or at variance with the temperature of the same sites recorded for normal or non-infected mice. It seems a reasonable probability that the temperature of an acutely inflamed area of the integument would be a few to several degrees higher than would obtain in normal non-inflamed tissue. Assuming this to be true, one must consider (1) if, after initial multiplication of the bacteria has succeeded in an environment having a temperature several degrees below that of the interior of the body, the organisms are capable, within limits, of adaptation for growth in temperatures somewhat higher, and (2) whether or not the temperature range for effective multiplication of *Myco. ulcerans in vivo* is within the limits of that in normal non-inflamed tissue and the maximum reached as a consequence of an inflammatory reaction. Conclusions regarding these factors must remain a matter of conjecture until precise information is available.

## Summary and Conclusions

To obtain information on the pathogenesis of infections with *Mycobacterium ulcerans* in mice, different groups of animals were inoculated intravenously via the tail vein, subcutaneously into the tail, into the periosteum of the left femur, and intracerebrally. The duration of the experiment was 120 days.

The most impressive features of the distribution of the lesions were the susceptibility of the soft structures of the tail and the rarity of lesions in the internal organs. In a few animals, acid-fast bacilli were observed within the caudal nerves, but there was no evidence that involvement of the nerves had a rôle in the initial stages of the pathogenesis of the infection. Lesions indicative of generalized infection were not observed.

It was recognized that the pathogenesis of *Myco. ulcerans* is dependent to a considerable extent on the temperature of the area where the bacteria are introduced or eventually find lodgment. Consequently, a study was undertaken in normal mice to determine the temperature of the tail, in contrast to that of the interior of the body as reflected by the temperature of the rectum. These temperatures were determined by means of thermistor-thermometers. The temperature determinations of the tail were approximately 11° to 12° C. lower than the temperatures recorded for the rectum and approximated that of the external environment.

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[ *Illustrations follow* ]

## LEGENDS FOR FIGURES

FIG. 1. Granulomatous nodule in lung of a mouse following experimental infection with *Mycobacterium ulcerans*. Animal died 75 days after intravenous inoculation. The black area in the central portion of the picture represents a closely packed accumulation of the bacteria. Tissue stained with carbolfuchsin. × 30.

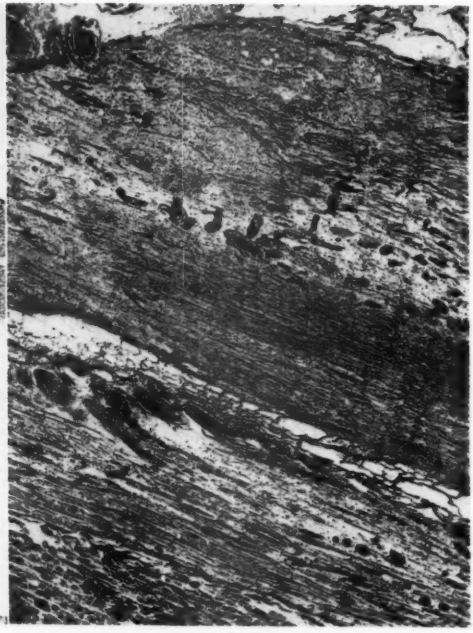
FIG. 2. Severe retrogressive changes in muscle overlying the femur of a mouse that died 67 days after experimental infection with *Myco. ulcerans*. Inoculum was placed into the periosteum. The myositis, which extended to the dermis, was severe and extensive. Dense accumulations of acid-fast bacillary forms were present in certain areas. For comparison with Figure 3. Hematoxylin and eosin stain. × 35.

FIG. 3. Muscle tissue over the femur from the same mouse as that from which Figure 2 was taken. Tissue stained with carbolfuchsin reveals compact accumulations of acid-fast bacillary cells, which photograph black. × 35.

FIG. 4. Large focal lesion in the cerebrum of a mouse inoculated intracerebrally with *Myco. ulcerans* and sacrificed 120 days later. Stained with carbolfuchsin to reveal acid-fast bacteria. The organisms, which are in dense masses, appear black in the central portion of the lesions. There are shattered fragments of calcium deposition at the periphery. × 35.

VOLTAGE

1



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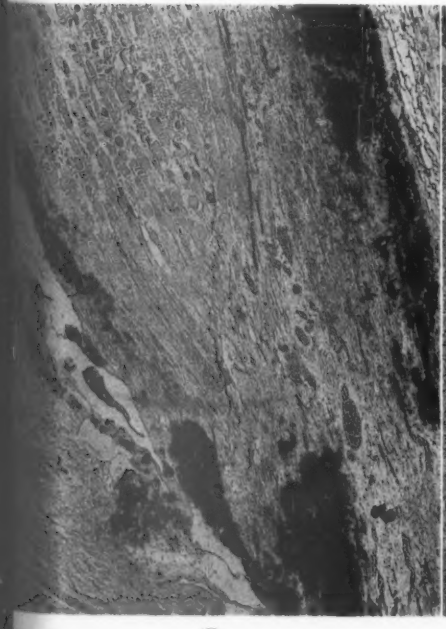

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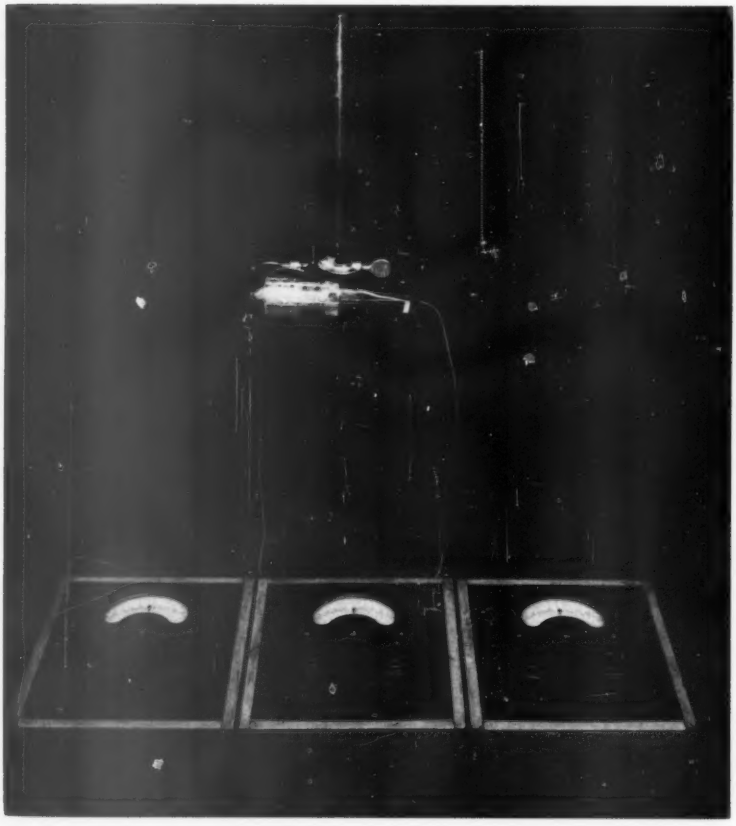
FIG. 5. Cross sectional view of the tail of a mouse inoculated intracerebrally with *Myco. ulcerans* and sacrificed after 120 days (same animal as that from which Fig. 4 was taken). Myeloid space occupied largely by dense accumulation of acid-fast bacteria, which appear dark or black in the photomicrograph. The infection appeared to be limited to the myeloid space. Tissue stained with carbolfuchsin. × 35.

FIG. 6. Arrangement of the apparatus used to measure simultaneously the temperature of the tail at two sites—proximal and distal—and the temperature in the rectum. Mouse may be noted in cage especially designed for immobilization of the animal during observation of temperatures.

5



6

# INCIDENCE IN NORMAL BREASTS OF FEATURES ASSOCIATED WITH CHRONIC CYSTIC MASTITIS *

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Anyone making a decision as to whether or not given histologic features, by themselves, constitute evidence of disease may be handicapped by failure to appreciate the range of normalcy of the tissue in question. This could be true of the mammary glands of women in the condition which has been termed chronic cystic mastitis.

For many decades, workers have studied the tissue changes related to multiple, benign, elusive, tender mammary swellings that often give rise to local pain and pressure. The enigma remains as to whether or not breasts that are normal to clinical examination should be considered diseased if they show histologic features qualitatively identical to those in breasts associated with a history and palpatory findings of chronic cystic mastitis. The purpose of this study was either to support or to refute the premise that the gross and microscopic differences between normal breasts and those with clinical features of chronic cystic mastitis are only of degree and not of quality.

## MATERIAL AND METHODS

The material providing the basis for this study consisted of both mammary glands obtained at necropsy from 100 women. No case was included in which there had been a complaint referable to the breasts at any time since puberty or in which any abnormality of the breasts had been detected on clinical examination. In order to obtain 100 cases fulfilling these criteria, a consecutive necropsy series of 125 women was required.

In all cases, both mammary glands were removed intact, weighed, and then fixed in formalin. Glands weighing less than 150 gm. were considered as being light in weight, those between 150 and 450 gm. were classed as moderate in weight, and those exceeding 450 gm. were considered heavy.

Each gland was cut into gross sections at intervals of 3 to 5 mm. in a plane at right angles to the ventrodorsal axis of the gland. The sec-

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tions were laid out in sequence and then examined. With the region of the nipple as a central point, all gross findings were listed in relation to their occurrence in one or more of the four quadrants of each gland.

Any tissue showing gross features not evident in a particular gland as a whole was removed for microtechnical processing. In addition, a minimum of one block of tissue was removed from the approximate center of each quadrant in every mammary gland. All blocks were labeled as to their exact sites of origin.

At least one paraffin section was prepared from each block, and all such sections were stained with hematoxylin and eosin. Other stains occasionally were used for additional sections. Microscopic findings were recorded as to their occurrence in particular quadrants of each breast.

## Results

Certain observations made on this material were categorized under the headings of (1) cysts and blunt-duct adenosis, (2) apocrine epithelium, (3) sclerosing adenosis, (4) intraductal epithelial hyperplasia, and (5) duct ectasia. All of these are features which by one name or another have received acceptance by a majority of writers as part of the complex of chronic cystic mastitis. The age distribution of three of these features is shown in Table I.

Table I

*Age Distribution of Certain Features of Chronic Cystic Mastitis in Clinically Normal Breasts*

| Group of patients | Age distribution, years | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 20–29 | 30–39 | 40–49 | 50–59 | 60–69 | 70–79 | 80–89 | 90–99 |
| Total series (100 patients) | 8 | 10 | 13 | 26 | 19 | 13 | 8 | 3 |
| Blunt-duct adenosis (63 patients) | 5 (8)* | 6 (9) | 10 (16) | 13 (21) | 14 (22) | 8 (13) | 7 (11) | 0 (0) |
| Apocrine epithelium (55 patients) | 5 (9) | 6 (11) | 7 (13) | 11 (20) | 11 (20) | 8 (14) | 6 (11) | 1 (2) |
| Intraductal epithelial hyperplasia (33 patients) | 0 (0) | 2 (6) | 4 (12) | 8 (24) | 5 (15) | 7 (21) | 4 (12) | 3 (9) |

* All numbers in parentheses are percentages of the particular group.

### *Cysts and Blunt-Duct Adenosis*

Included under the heading of cysts and blunt-duct adenosis were epithelial aggregates clearly fitting the description of Foote and Stewart.[1] These aggregates apparently are identical with the bunches of

small mammary cysts demonstrated by Ingleby[2] and with the cysts of ductal origin described by Semb.[3]

Blunt-duct adenosis was found in 63 of the 100 patients in our series. It was present in both breasts in 34 of these patients. The right breast only was involved in 15 of the remaining 29 patients, whereas the left breast only was involved in 14. The distribution of blunt-duct adenosis according to quadrants of the breasts in these 63 patients is given in Table II. The relationships of blunt-duct adenosis to the weight of the mammary glands and to the relative amounts of glandular substance and fatty tissue are summarized in Tables III and IV.

TABLE II

*Distribution of Blunt-Duct Adenosis: 63 Cases*

| Quadrant of breast involved | Cases |
|---|---|
| Right upper outer | 23 |
| Right lower outer | 22 |
| Right upper inner | 25 |
| Right lower inner | 14 |
| Left upper outer | 25 |
| Left lower outer | 21 |
| Left upper inner | 20 |
| Left lower inner | 23 |

TABLE III

*Relationship of Weight of the Breast to Certain Features of Chronic Cystic Mastitis in Clinically Normal Breasts*

| Group of patients | Weight of breast | | |
|---|---|---|---|
| | Heavy | Moderate | Light |
| Total series (100 patients) | 13 | 46 | 41 |
| Blunt-duct adenosis (63 patients) | 7 (11)* | 31 (49) | 25 (40) |
| Apocrine epithelium (55 patients) | 7 (13) | 26 (47) | 22 (40) |
| Intraductal epithelial hyperplasia (33 patients) | 4 (12) | 17 (52) | 12 (36) |

* All numbers in parentheses are percentages of the particular group.

Gross evidence of blunt-duct adenosis was noted in 31 of the 63 patients (49 per cent), whereas evidence of blunt-duct adenosis was forthcoming only on microscopic examination in the remaining 32 patients (51 per cent).

Histologically, the gland-like spaces in a given zone of adenosis were lined by epithelium closely resembling that of normal mammary ducts in 30 of the 63 patients (Fig. 1). In 43 patients, only epithelium of the apocrine variety was found in given zones of blunt-duct adenosis (Fig. 2). Both types of epithelium occurred together in given zones of adenosis in the same breast in 13 patients.

TABLE IV

*Relationship of Amount of Non-adipose Tissue to Certain Features of Chronic Cystic Mastitis in Clinically Normal Breasts*

| Group of patients | Relative amount of non-adipose tissue | | |
|---|---|---|---|
| | Abundant | Moderate | Small |
| Total series (100 patients) | 20 | 26 | 54 |
| Blunt-duct adenosis (63 patients) | 17 (27)* | 15 (24) | 31 (49) |
| Apocrine epithelium (55 patients) | 14 (25) | 13 (24) | 28 (51) |
| Intraductal epithelial hyperplasia (33 patients) | 6 (18) | 9 (27) | 18 (55) |

* All numbers in parentheses are percentages of the particular group.

Most commonly, the adenoid spaces of blunt-duct adenosis contained only albuminous material. In other instances, the content was mucus. Least commonly, a mixture of these two substances was found within a single epithelium-lined space.

### *Apocrine Epithelium*

A total of 55 patients in this study had apocrine epithelium in their breasts. The age distribution of these patients is shown in Table I.

This feature of chronic cystic mastitis was present in both breasts in 40 per cent of the patients, whereas it was present only on the right side in 38 per cent and only on the left side in 22 per cent. This distribution of apocrine epithelium according to quadrants of the breasts in these 55 patients is given in Table V. Apocrine epithelium was observed in zones of blunt-duct adenosis in 42 patients, as part of the lining of a duct in 18 patients (Fig. 3) and as part of the epithelium of one or more lobules in eight.

TABLE V

*Distribution of Mammary Apocrine Epithelium: 55 Cases*

| Quadrant of breast involved | Cases |
|---|---|
| Right upper outer | 17 |
| Right lower outer | 20 |
| Right upper inner | 18 |
| Right lower inner | 13 |
| Left upper outer | 11 |
| Left lower outer | 18 |
| Left upper inner | 15 |
| Left lower inner | 13 |

The relationships of the weight of the mammary glands and of the relative amounts of non-adipose and adipose tissue in the glands to the presence of apocrine epithelium are summarized in Tables III and IV.

### *Sclerosing Adenosis*

Identification of sclerosing adenosis in this study was made on the basis of the descriptions provided by Foote and Stewart,[1] Urban and Adair,[4] and Heller and Fleming.[5] A zone of sclerosing adenosis found in this study is illustrated in Figure 4.

Sclerosing adenosis was found in but nine of the 100 patients studied. Only a single area of involvement was found in the breasts of five patients, whereas multiple sites were present in the breasts of four. A predilection for a particular quadrant or quadrants of the breast was not apparent. The occurrence of sclerosing adenosis appeared to bear no particular relationship to that of blunt-duct adenosis.

### *Intraductal Epithelial Hyperplasia*

Included in the category of intraductal epithelial hyperplasia were papillary, cribriform, and solid types of hyperplasia of ductal epithelium. The minimal degree of hyperplasia for inclusion in this study was the formation of an epithelial eminence that reached at least half way across the lumen of a duct when sectioned at right angles to the long axis of the duct. Any epithelial formation within a duct that possessed supportive fibrous connective tissue or that contained vascular channels was considered to be a papilloma and was not included.

A total of 33 of these 100 patients had intraductal epithelial hyperplasia of the breast. The age distribution is set forth in Table I. Both breasts were the site of this finding in 11 of the 33 patients, whereas

TABLE VI

*Distribution of Intraductal Epithelial Hyperplasia: 33 Cases*

| Quadrant of breast involved | Cases |
|---|---|
| Right upper outer | 7 |
| Right lower outer | 6 |
| Right upper inner | 8 |
| Right lower inner | 7 |
| Left upper outer | 8 |
| Left lower outer | 6 |
| Left upper inner | 6 |
| Left lower inner | 4 |

TABLE VII

*Distribution of Duct Ectasia: 17 Cases*

| Quadrant of breast involved | Cases |
|---|---|
| Right upper outer | 12 |
| Right lower outer | 13 |
| Right upper inner | 13 |
| Right lower inner | 13 |
| Left upper outer | 9 |
| Left lower outer | 9 |
| Left upper inner | 9 |
| Left lower inner | 12 |

only the right breast was affected in 12 patients and only the left breast in 10.

A single quadrant of the breast was involved in 21 patients, whereas

hyperplasia was found in multiple quadrants in 12 patients. The distribution according to quadrants is summarized in Table VI.

The relationships of the weight of the breasts and of the relative amounts of adipose and non-adipose tissue to the presence of intraductal epithelial hyperplasia are shown in Tables III and IV.

Intraductal epithelial hyperplasia was 25 times more common in small excretory ducts than it was in large ducts. The term small was used arbitrarily to indicate ducts with a caliber of less than 500 $\mu$, whereas large ducts were 500 $\mu$ or more in caliber.

### *Duct Ectasia*

In no instance in our study was a duct considered to be dilated unless its lumen measured at least 3 mm. in diameter and could be followed in this caliber for a distance of at least 10 mm. (Fig. 5). Dilatation of the ductal system meeting these requirements was present in 17 of these 100 cases.

With regard to the right breast only, dilated ducts were observed in all four quadrants in 12 instances; involvement of two quadrants was noted in one case, and only a single quadrant was affected in one. Essentially the same findings were noted in the left breast, with all four quadrants being involved in the majority of instances. The distribution of duct ectasia according to quadrants is seen in Table VII. The presence or absence of blunt-duct adenosis appeared to bear no relationship to the occurrence of duct ectasia.

## Comment

Short of their universal occurrence, it is impossible to state how commonly the various features associated with chronic cystic mastitis must be found in clinically normal breasts in order to be assured that these phenomena are qualitatively normal. Perhaps their universal occurrence may be suspected, for a greater incidence goes hand in hand with what appears to be more extensive histologic sampling. If blunt-duct adenosis is taken as an example, Semb's[3] series of 32 cases contained but three (9 per cent) in which one or both breasts showed what probably represented blunt-duct adenosis. Lindgren's[6] study, carried out later, in which breasts of 60 women (120 breasts) were studied, showed what apparently was blunt-duct adenosis in 16 glands. The changes were bilateral in all these cases, giving an incidence for the series of 13 per cent. The report of Frantz and associates[7] showed that cystic disease, which we assume to be synonymous with blunt-duct adenosis, was present in a larger percentage of cases than was reported

by Lindgren but in a smaller percentage than we noted. The observed occurrence of apocrine epithelium and intraductal epithelial hyperplasia has shown a similar trend.

That the breasts of all women are so disposed, however, still awaits demonstration rather than assumption. Unhappily, such demonstration may require serial blocking of the tissue, and any study of a sizable series of cases utilizing such a method would be of forbidding proportions.

Nevertheless, it is apparent that certain of the tissue changes noted in this study occurred so commonly that their mere presence would not warrant the diagnosis of chronic cystic mastitis. The burden of proof appears to lie with those who claim that the presence of such changes is indicative of abnormality. These features are blunt-duct adenosis, the presence of apocrine epithelium and intraductal epithelial hyperplasia.

This study adds one observation that might help solve the controversial relationship between chronic cystic mastitis and mammary carcinoma. Carcinoma of the breast arises with disproportionate frequency in the upper outer quadrant. On the other hand, this investigation has shown that tissue changes commonly accepted as representing chronic cystic mastitis have a surprisingly even qualitative distribution among the various quadrants.

## Summary and Conclusions

The mammary glands of 100 women were obtained at necropsy and studied grossly and microscopically. All these breasts had been considered as entirely normal on clinical examination. A high incidence of certain of the tissue changes considered to be part of the complex of chronic cystic mastitis was disclosed. Thus, it is concluded that the mere qualitative presence of blunt-duct adenosis, apocrine epithelium, and intraductal epithelial hyperplasia in the breasts of women is insufficient to warrant such tissue being considered as diseased.

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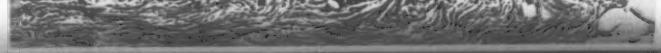
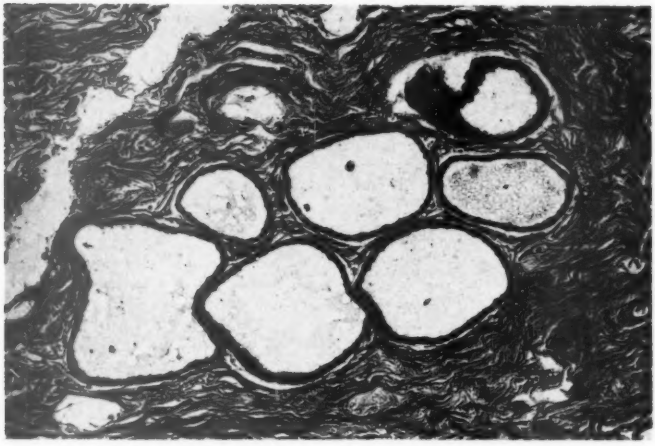
## LEGENDS FOR FIGURES

FIG. 1. Blunt-duct adenosis. The spaces are lined by attenuated epithelium of the ordinary ductal type. Hematoxylin and eosin stain. × 100.

FIG. 2. Blunt-duct adenosis. The lining epithelium is of the apocrine type. Hematoxylin and eosin stain. × 175.

FIG. 3. Papillary hyperplasia of apocrine epithelium in a mammary glandular duct. Hematoxylin and eosin stain. × 300.





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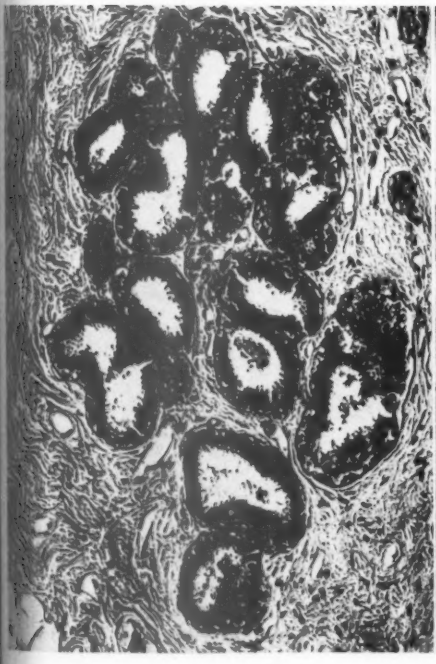

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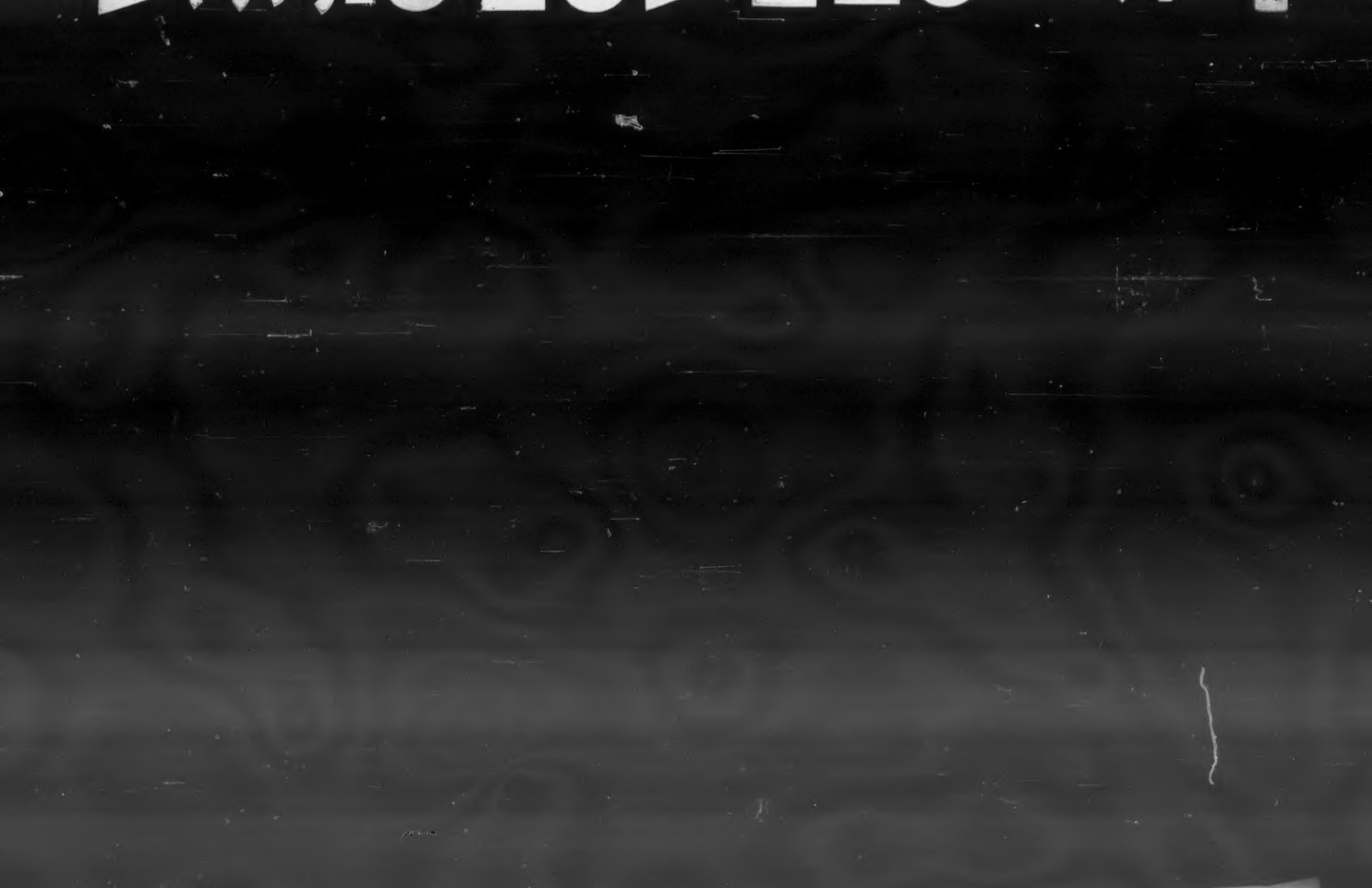
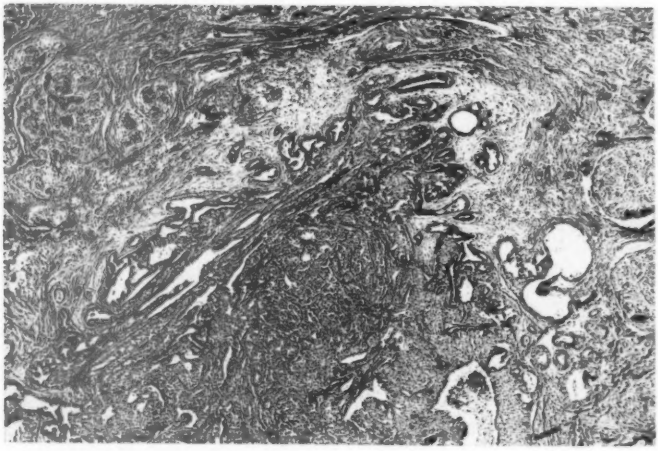
FIG. 4. Sclerosing adenosis found in a clinically normal breast. Hematoxylin and eosin stain. × 30.

FIG. 5. Duct ectasia involving a single quadrant of a clinically normal breast. Hematoxylin and eosin stain. × 4½.





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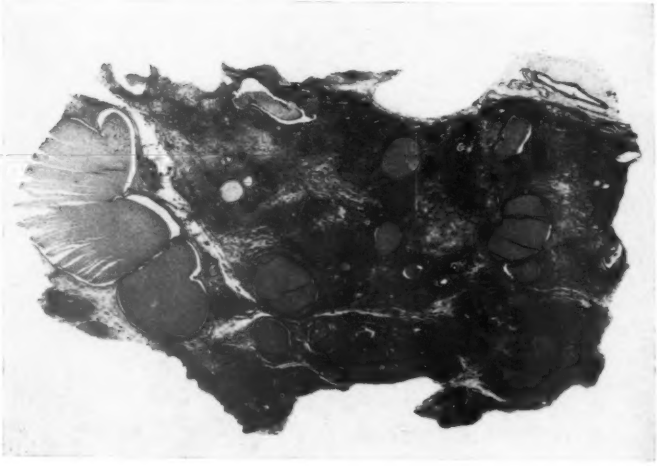

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# INFLUENCE OF SUBCUTANEOUSLY ADMINISTERED BOVINE GLOBULIN ON THE RESPONSE OF RABBITS TO INTRAVENOUS GRAM-NEGATIVE ENDOTOXIN OR LIQUOID *

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Many human diseases are characterized by the presence of vascular fibrinoid lesions. These diseases, known as connective tissue diseases,[1] collagen diseases,[2] or systemic fibrinoid diseases,[3] include rheumatic fever, rheumatoid arthritis, polyarteritis, dermatomyositis, scleroderma, disseminated lupus erythematosus, and thrombotic thrombocytopenic purpura.

The common characteristic alteration of this group of systemic diseases is the presence of diffuse vascular fibrinoid lesions, associated with areas of hemorrhage and necrosis in many organs. One central problem in the pathogenesis of these diseases is the origin and nature of this fibrinoid material. Present evidence strongly suggests that this material, or its precursors, is derived from altered or abnormal plasma proteins, and that it is deposited in sites of structural or functional vascular alterations.[3-8]

Rich,[9] in 1942, suggested that hypersensitivity might play a rôle in the development of the fibrinoid lesions of polyarteritis. This suggestion was followed by numerous attempts to reproduce the lesions in rabbits.[10-20] In these experiments, rabbits were given a variety of foreign proteins such as horse serum,[11-17] horse serum and egg albumin,[18] or bovine gamma globulin[10,19,20] as well as other mixtures. These substances were given intravenously,[10-17,19,20] intraperitoneally,[18] subcutaneously,[18] or by combinations of these methods.[18] The number of injections varied from one[10] to 130,[18] and the total amount of protein given ranged from 700 mg. to 71.5 gm. per kg. of body weight.

A variety of lesions was described in the reports dealing with these experiments. Some workers described cellular coronary arteritis which was present in from 30 to 85 per cent of the animals, and occasional fibrinoid-containing lesions were described. Other investigators reported "granulomatous" lesions of the cardiac valves, aortitis, pulmo-

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nary thrombi or emboli, chronic glomerular endothelial proliferation, and generalized arteritis. Other attempts to reproduce these experimental lesions were unsuccessful.[11]

In previous studies, it has been shown that fibrinoid lesions may be produced regularly in rabbits by two properly spaced intravenous injections of Gram-negative endotoxin[4] or by a single injection of endotoxin in conjunction with the high molecular weight acidic polymer, sodium polyanethol sulfonate (Liquoid).[5] In other studies it was shown that similar lesions could be produced by a single injection of endotoxin in rabbits given cortisone,[21] colloidal iron or carbon,[22] or Thorotrast or trypan blue.[23] These observations suggested that many different substances might duplicate or mimic the action of an intravenous injection of endotoxin, and "prepare" the cardiovascular system for the deposition of fibrinoid. In an effort to test this hypothesis, rabbits were given subcutaneous injections of bovine gamma globulin, followed by an intravenous injection of Gram-negative endotoxin or sodium polyanethol sulfonate (Liquoid). The present paper is concerned with the changes produced by these procedures.

## Materials and Methods

One hundred sixty-eight hybrid albino rabbits of mixed sexes, weighing 1 to 1.5 kg., were used in the experiments. They were fed Purina rabbit pellets and had free access to water. Following death or sacrifice of the animals, postmortem examinations were performed and the tissues were fixed in 10 per cent neutral formalin. Sections were taken routinely from the kidneys, heart, spleen, pancreas, liver, adrenal glands, and lungs. Hematoxylin and eosin were used routinely. Selected sections were stained by the periodic acid-Schiff method and by a modified form of Movat's Pentachrome I stain.[24] In this modification, the Weigert-Hart resorcin-fuchsin and the alcoholic saffron steps were omitted. By this trichrome method, fibrinoid material stained a brilliant red, and was easily separated from the erythrocytes, which stained reddish brown.

Meningococcal endotoxin, prepared by a method described previously,[25] was used in certain experiments. It was diluted 1:80 with sterile, pyrogen-free, isotonic saline solution and injected into the marginal ear vein of the animals in a volume of 2 cc. *Escherichia coli* endotoxin* (lipopolysaccharide O-111:B4, lot no. B35527) was injected into the marginal ear vein in a concentration of 500 μg. per cc. in sterile, pyrogen-free, isotonic saline solution. Sodium polyanethol-

* Generously supplied by Difco Laboratories, Detroit, Mich.

sulfonate (Liquoid)* was dissolved in sterile, isotonic saline solution, passed through a Seitz filter, and injected in dosages of 10 mg. into the marginal ear vein.

Three commercial preparations of bovine gamma globulins† were used in the experiments. Each was dissolved in sterile, isotonic saline solution in a concentration of 10 gm. per cent. One-half cc. of the globulin solution was injected subcutaneously over the abdomen daily for a period of 6 days. Seventy-two hours following the last injection of globulin, a single intravenous injection of endotoxin or 10 mg. of Liquoid was given intravenously. The animals died or were sacrificed 48 hours after the administration of toxin or Liquoid.

Serial blood samples were drawn from 77 of the animals and the proteins studied by paper electrophoresis. Total serum protein determinations were carried out on all samples by the Weichselbaum biuret method,[26] using a Bausch and Lomb colorimeter. Paper electrophoretic patterns were determined on a Spinco model R paper electrophoresis system, using B-2 buffer (ionic strength 0.075) with a 16 hour migration period at 5 ma. per cell. The paper strips were stained with Spinco B-1 dye, composed of bromphenol blue and zinc sulfate. These were analyzed on the Spinco analytrol using a B-3 cam. Prior to the use of paper electrophoresis, the total serum proteins and albumin-globulin ratio of 35 of the animals were determined by the Weichselbaum biuret method.

## Results

The numbers of animals used in the various experiments, and the results, are summarized in Tables I to V. As shown there, the lethal effects of the various commercial preparations of globulin given in association with endotoxin varied considerably. It may be seen, for example, that 84 per cent of the animals given Pentex fraction II died within 48 hours after an injection of *E. coli* endotoxin, but that only 20 per cent of the animals given NBC fraction II and *E. coli* toxin died within an equivalent period of time. It would also appear that the mortality rate might be affected by the type of endotoxin given, but this difference between meningococcal toxin and *E. coli* toxin may be more apparent than real. It has been observed in other studies that the potency of different preparations of crude meningococcal toxin varies considerably, and this factor may account for the difference in the lethal effects of the two types of endotoxin used in

* Obtained from Hoffmann-La Roche, Inc., Nutley, N.J.

† Obtained from Armour Laboratories, Armour & Company, Kankakee, Ill. (Fraction II); Pentex, Inc., Kankakee, Ill. (Fraction II and mixed gamma globulin); and Nutritional Biochemical Corporation, Cleveland, Ohio (Fraction II).

TABLE I

*Incidence of Lesions Following Administration of Bovine Gamma Globulin (Fraction II) from Nutritional Biochemical Corporation*

| Intravenous injection | Number of animals | Number dying in less than 48 hours | Microscopic lesions | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Renal | Cardiac | | Spleen | Liver | Lung |
| | | | | Cellular | Fibrinoid | | | |
| *Escherichia coli* endotoxin (500 μg.) | 25 | 5 | 19 | 12 | 6 | 13 | 9 | 7 |
| Liquoid (10 mg.) | 10 | 0 | 4 | 1 | 0 | 3 | 2 | 0 |
| None | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

TABLE II

*Incidence of Lesions Following Administration of Bovine Gamma Globulin (Fraction II) from Pentex, Incorporated*

| Intravenous injection | Number of animals | Number dying in less than 48 hours | Microscopic lesions | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Renal | Cardiac | | Spleen | Liver | Lung |
| | | | | Cellular | Fibrinoid | | | |
| *Escherichia coli* endotoxin (500 μg.) | 13 | 11 | 10 | 7 | 7 | 12 | 8 | 6 |
| Meningococcal toxin (2 cc. 1:80 dilution) | 13 | 0 | 9 | 9 | 4 | 3 | 4 | 1 |
| Liquoid (10 mg.) | 22 | 0 | 6 | 10 | 3 | 5 | 0 | 0 |

these studies. It is obvious from these tables that, in all of the experiments in which Liquoid was given, there was a low mortality rate.

No good correlation existed between the lethal effects and the incidence of morphologic changes. In the animals which died within 48 hours following administration of endotoxin, for example, 69 per cent were found to have renal lesions, while in those which were sacrificed 48 hours after the administration of endotoxin, renal lesions were present in 61 per cent.

### *Morphologic Changes*

Lesions involving the kidneys, heart, spleen, liver, and lungs were consistently observed. The incidence of such lesions varied somewhat depending on the preparation of globulin used and the material given intravenously. The incidence of the lesions observed after each type of globulin is summarized in Tables I to IV. Since the morphologic changes were basically similar in each group, a summary is shown in Table V.

Grossly, the kidneys were pale and swollen, and numerous petechiae were scattered over the capsular surface. In 13 per cent of the rabbits given endotoxin and in 5 per cent of those given Liquoid, bilateral cortical necrosis was observed. This was similar to the lesion described in rabbits given two properly spaced intravenous injections of endotoxin,[4] or a single injection of endotoxin in association with the administration of Liquoid.[5]

Microscopic examination showed the presence of hyaline fibrinoid material in the glomerular capillaries. This material was present in varying numbers of glomeruli and usually did not involve all of the capillaries of any one glomerulus. Some of the glomeruli appeared almost completely occluded, while in others only small droplets of the material were present (Fig. 1). In many glomeruli, the appearance of this material was similar to that seen in diabetic glomerulosclerosis, but it was not present in the afferent arterioles. The material was similar in morphologic and tinctorial properties to that described in previous papers.[3-6] In occasional kidneys it was noted that fibrinoid was deposited in the media and, less commonly, the perivascular spaces of large arteries. Considerable perivascular edema and minimal lymphocytic cellular reaction were present also (Figs. 2 and 3).

In addition to the presence of fibrinoid, the glomeruli were uniformly enlarged and hypercellular. The latter appeared to be due to enlargement and proliferation of the endothelial cells (Fig. 4).

Aside from areas of hemorrhage, no consistent gross changes were observed in the heart. Microscopically, areas of muscle necrosis,

TABLE III

*Incidence of Lesions Following Administration of Bovine Gamma Globulin (Mixed) from Pentex, Incorporated*

| Intravenous injection | Number of animals | Number dying in less than 48 hours | Microscopic lesions | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Renal | Cardiac | | Spleen | Liver | Lung |
| | | | | Cellular | Fibrinoid | | | |
| Meningococcal toxin (2 cc. 1:80 dilution) | 10 | 1 | 3 | 7 | 1 | 5 | 1 | 0 |
| Liquoid (10 mg.) | 10 | 0 | 2 | 3 | 0 | 3 | 1 | 2 |
| None | 9 | 0 | 4 | 2 | 0 | 0 | 0 | 0 |

TABLE IV

*Incidence of Lesions Following Administration of Bovine Gamma Globulin (Fraction II) from Armour Laboratories*

| Intravenous injection | Number of animals | Number dying in less than 48 hours | Microscopic lesions | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Renal | Cardiac | | Spleen | Liver | Lung |
| | | | | Cellular | Fibrinoid | | | |
| *Escherichia coli* toxin (500 μg.) | 8 | 4 | 7 | 7 | 1 | 8 | 0 | 2 |
| Meningococcal toxin (2 cc. 1:80 dilution) | 13 | 10 | 12 | 8 | 7 | 1 | 6 | 10 |
| Liquoid (10 mg.) | 22 | 2 | 15 | 12 | 4 | 5 | 2 | 5 |
| None | 8 | 0 | 2 | 0 | 0 | 0 | 0 | 2 |

mononuclear and heterophilic cellular reaction, hemorrhage, and calcification were observed commonly. In an occasional animal extensive areas of ischemic necrosis, with a pronounced heterophilic cellular reaction, were noted. Valvular lesions which consisted of hemorrhage, edema, and mononuclear cellular reaction were observed in many of the animals.

In addition to these lesions, fibrinoid material was present beneath the endothelium of the coronary arteries or aorta, and within the substance of the valves. This material was often associated with a marked cellular reaction which consisted of heterophil, mononuclear, and Anitschkow cells (Figs. 5 to 7). In control animals given only globulin, somewhat similar inflammatory arterial lesions were observed in 9 per cent, but no fibrinoid was noted.

Gross changes in the spleen were observed in only 5 per cent of the animals. These consisted of enlargement, increased consistency, and infarcts. Microscopically, fibrinoid material was observed in the splenic sinusoids in varying quantities. In some, the sinusoids appeared completely filled and there were associated areas of necrosis, while in others small clumps of the material were scattered at random through the sinusoids.

No gross changes were observed in the liver. Extensive areas of hemorrhage and necrosis were

TABLE V

*Summary of Incidence of Lesions Following Administration of Bovine Gamma Globulin*

| Intravenous injection | Number of animals | Number dying in less than 48 hours | Microscopic lesions | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Renal | Cardiac | | Spleen | Liver | Lung |
| | | | | Cellular | Fibrinoid | | | |
| *Escherichia coli* toxin (500 μg.) | 46 | 20 | 36 | 26 | 14 | 33 | 17 | 15 |
| Meningococcal toxin (2 cc. 1:80 dilution) | 36 | 11 | 24 | 24 | 13 | 19 | 11 | 11 |
| Liquoid (10 mg.) | 64 | 2 | 27 | 26 | 7 | 16 | 5 | 7 |
| None | 22 | 0 | 6 | 2 | 0 | 0 | 0 | 2 |

noted microscopically in approximately 32 per cent of the animals given endotoxin and in 8 per cent of those given Liquoid. In association with the necrotic areas, hyaline fibrinoid thrombi were observed occasionally in the efferent veins (Fig. 8).

Microscopic sections from the lungs showed the presence of hyaline thrombi or emboli in the pulmonary arteries in approximately 30 per cent of the animals given endotoxin but in only 3 per cent of those given Liquoid. These thrombi or emboli were occasionally covered by endothelium and usually contained an admixture of erythrocytes and leukocytes. Fibrinoid material was observed also beneath the pulmonary arterial endothelium in a few of the animals.

### *Changes in Serum Proteins*

During the course of the experiments, initial studies of serum proteins were done by the Weichselbaum biuret method[26] to determine the albumin-globulin ratio. Thirty-five animals were studied by this method. These determinations showed that there was a shift in the albumin-globulin ratio from a normal range of 3.0 to 1.5 to ratios ranging from 1.2 to 0.65. In an effort to evaluate these changes, further studies were carried out by means of paper electrophoresis.

TABLE VI

*Average Serum Protein Values Determined by Paper Electrophoresis of Control Rabbits*

| Fractions | Normal | 72 hours after last globulin | Death (48 hours after previous sample) |
|---|---|---|---|
| Total protein | 4.32 ±0.46 | 4.13 ±0.43 | 4.28 ±0.44 |
| Albumin-globulin ratio | 2.11 ±0.73 | 1.07 ±0.42 | 2.10 ±0.68 |
| Albumin | 2.93 ±0.21 | 2.14 ±0.39 | 2.90 ±0.47 |
| Alpha$_1$ globulin | 0.36 ±0.12 | 0.36 ±0.14 | 0.23 ±0.07 |
| Alpha$_2$ globulin | 0.29 ±0.06 | 0.35 ±0.12 | 0.19 ±0.06 |
| Beta globulin | 0.39 ±0.08 | 0.45 ±0.12 | 0.36 ±0.25 |
| Para-beta globulin | 0.063 ±0.002 | 0.166 ±0.002 | 0.082 ±0.002 |
| Gamma globulin | 0.29 ±0.22 | 0.66 ±0.24 | 0.55 ±0.35 |
| Number of animals | 8 | 8 | 8 |

± Values are the standard deviations.

TABLE VII

*Average Serum Protein Values Determined by Paper Electrophoresis in Rabbits Given Endotoxin After Globulin*

| | With microscopic fibrinoid lesions | | | Without microscopic fibrinoid lesions | | |
|---|---|---|---|---|---|---|
| Fractions | Normal | 72 hours after last globulin | Death (48 hours after endotoxin) | Normal | 72 hours after last globulin | Death (48 hours after endotoxin) |
| Total proteins | 4.46 ±0.73 | 4.77 ±0.73 | 4.48 ±0.97 | 3.93 ±0.52 | 4.55 ±0.52 | 4.72 ±0.60 |
| Albumin-globulin ratio | 2.06 ±0.95 | 1.37 ±0.42 | 1.13 ±0.73 | 2.17 ±0.95 | 1.35 ±0.39 | 1.45 ±0.19 |
| Albumin | 3.00 ±0.38 | 2.76 ±0.45 | 2.38 ±0.75 | 2.69 ±0.24 | 2.61 ±0.45 | 2.78 ±0.36 |
| Alpha$_1$ globulin | 0.31 ±0.08 | 0.43 ±0.05 | 0.39 ±0.14 | 0.28 ±0.12 | 0.38 ±0.06 | 0.40 ±0.08 |
| Alpha$_2$ globulin | 0.29 ±0.07 | 0.40 ±0.10 | 0.40 ±0.11 | 0.23 ±0.06 | 0.36 ±0.04 | 0.42 ±0.09 |
| Beta globulin | 0.46 ±0.18 | 0.39 ±0.12 | 0.49 ±0.16 | 0.37 ±0.18 | 0.45 ±0.08 | 0.50 ±0.13 |
| Para-beta globulin | 0.037 ±0.002 | 0.158 ±0.002 | 0.188 ±0.003 | 0.074 ±0.003 | 0.142 ±0.002 | 0.076 ±0.002 |
| Gamma globulin | 0.36 ±0.30 | 0.63 ±0.25 | 0.63 ±0.33 | 0.29 ±0.14 | 0.61 ±0.21 | 0.54 ±0.24 |
| Number of animals | 17 | 17 | 17 | 7 | 7 | 7 |

± Values are the standard deviations.

In 77 animals, serum was studied before the injections of globulin were begun, 72 hours after the last injection of globulin, and at the time of sacrifice (48 hours after injection of endotoxin or Liquoid). Of the total, 42 were given endotoxin after globulin, 27 were given Liquoid, and 8 were given only globulin. The results of these studies are summarized in Tables VI, VII, and VIII.

As shown in the tables, in approximately 33 per cent of the animals there was a reversal of the albumin-globulin ratio 72 hours after the last injection of globulin, without a decrease in absolute concentration of albumin. In approximately 26 per cent of the animals, normal patterns showed the presence of a para-beta globulin, which migrated between the beta and gamma globulin bands. Seventy-two hours after the last globulin injection, this fraction was present in approximately 93 per cent of the rabbits studied. If calculated as part of the beta globulin component, this fraction made up more than 20 per cent of the beta globulin in approximately 80 per cent of the rabbits.

Although no definite correlation could be shown between the changes in serum proteins and the development of morphologic changes, the following points are of interest. Of the animals in which para-beta globulin was present 72 hours after the final (sixth) injection of bovine globulin, there was a continued increase in this fraction in the animals given an intravenous injection of Liquoid. Of those given an injection of endotoxin, this fraction increased only in those which developed fibrinoid lesions. In the control animals given only globulin, and in those which failed to develop fibrinoid lesions after an injection of endotoxin, the amount of para-beta globulin decreased. The changes in the albumin-globulin ratio and in the gamma globulin levels were similar to that of the para-beta globulin.

## Discussion

The occurrence of diffuse lesions in rabbits given Gram-negative endotoxin, or endotoxin in conjunction with the administration of Liquoid, colloidal iron, Thorotrast, or cortisone, has been described in previous papers.[4,5,21-23] These lesions are characterized by the presence of hyaline fibrinoid material in the renal glomerular capillaries, splenic sinusoids, pulmonary arteries, beneath the endothelium of the coronary arteries, and within the substance of the cardiac valves. One of the characteristic gross lesions which occurs with the experimental procedures mentioned above is bilateral renal cortical necrosis, due to glomerular capillary occlusion by massive deposits of fibrinoid material. These diffuse fibrinoid lesions comprise the reaction known as the generalized Shwartzman phenomenon.

TABLE VIII

*Average Serum Protein Values Determined by Paper Electrophoresis in Rabbits Given Liquoid After Globulin*

| | With microscopic fibrinoid lesions | | | Without microscopic fibrinoid lesions | | |
|---|---|---|---|---|---|---|
| Fractions | Normal | 72 hours after last globulin | Death (48 hours after Liquoid) | Normal | 72 hours after last globulin | Death (48 hours after Liquoid) |
| Total protein | 4.25 ±0.67 | 4.57 ±0.69 | 4.89 ±0.70 | 4.04 ±0.46 | 4.45 ±0.59 | 4.51 ±1.10 |
| Albumin-globulin ratio | 1.76 ±0.86 | 1.44 ±0.47 | 1.01 ±0.36 | 2.06 ±0.49 | 1.43 ±0.27 | 0.99 ±0.17 |
| Albumin | 2.71 ±0.31 | 2.70 ±0.46 | 2.46 ±0.47 | 2.72 ±0.24 | 2.62 ±0.34 | 2.24 ±0.60 |
| Alpha$_1$ globulin | 0.38 ±0.11 | 0.34 ±0.12 | 0.39 ±0.13 | 0.31 ±0.09 | 0.41 ±0.06 | 0.39 ±0.11 |
| Alpha$_2$ globulin | 0.32 ±0.09 | 0.32 ±0.08 | 0.41 ±0.08 | 0.25 ±0.06 | 0.33 ±0.07 | 0.47 ±0.16 |
| Beta globulin | 0.43 ±0.11 | 0.44 ±0.12 | 0.67 ±0.26 | 0.45 ±0.12 | 0.36 ±0.09 | 0.54 ±0.17 |
| Para-beta globulin | 0.056 ±0.003 | 0.154 ±0.003 | 0.178 ±0.004 | 0.050 ±0.003 | 0.168 ±0.002 | 0.231 ±0.003 |
| Gamma globulin | 0.35 ±0.26 | 0.62 ±0.25 | 0.78 ±0.37 | 0.26 ±0.15 | 0.56 ±0.17 | 0.64 ±0.27 |
| Number of animals | 13 | 13 | 13 | 12 | 12 | 12 |

± Values are the standard deviations.

In the present studies it has been shown that fibrinoid lesions may be produced in rabbits given a foreign protein subcutaneously, followed by an intravenous injection of endotoxin or Liquoid. Although these lesions are fundamentally similar to those which occur in the generalized Shwartzman phenomenon, there are certain noteworthy differences. The number of renal glomeruli involved, the focal or segmental glomerular fibrinoid deposits, and the presence of fibrinoid material in the larger renal arteries differ from the renal lesion of the Shwartzman phenomenon. Likewise the glomerular endothelial proliferation and the inflammatory reaction noted about the fibrinoid lesions of the coronary arteries are differences which separate the lesions from those of the conventional Shwartzman type.

The accumulations of fibrinoid in the splenic sinusoids, the presence of hyaline thrombi or emboli in the pulmonary arteries, and the occurrence of areas of hepatic necrosis, however, are strikingly similar to the lesions of the Shwartzman phenomenon. These observations suggested that further information concerning the pathogenesis of the lesions might be obtained by a study of the serum proteins.

These were studied by chemical means and by serial paper electrophoresis. Definite alterations were observed in the electrophoretic patterns in all animals 72 hours after the sixth subcutaneous injection of globulin. These changes included a shift in the albumin-globulin ratio, the appearance of and increased amounts of a para-beta globulin, and an increase in the gamma globulin. Since no regular decrease in the serum albumin level was observed, the change in the albumin-globulin ratio appears to be explained by the increased para-beta globulin and gamma globulin.

In control animals given only globulin (Table VI), electrophoretic studies 120 hours after the final injection of globulin showed a return toward a normal pattern. It is of interest that in the rabbits given endotoxin, and in which no fibrinoid lesions were observed, a similar return to a normal pattern was observed. Of the animals given endotoxin in which fibrinoid lesions developed, and in all the rabbits given Liquoid, persistent alterations were observed in the serum electrophoretic patterns 48 hours after the administration of toxin or Liquoid (Tables VII and VIII). The significance of these changes is not known at present. The results suggest, however, that the administration of Liquoid may provide a continued stimulus for the production of altered serum proteins, or increased amounts of normal serum proteins. No definite correlation could be shown between changes in serum proteins and the presence of fibrinoid lesions, although the time between the

administration of Liquoid or endotoxin and the determination of serum proteins is of such an interval (48 hours) that an acute change would not have been detected.

It is known that the administration of endotoxin to rabbits is followed by the appearance of a cold heparin-precipitable fibrinogen in the plasma.[7] This altered fibrinogen appears to be involved in the formation of fibrinoid material, and changes in the amount of this material can be correlated with the development of fibrinoid lesions.[8] In the present studies the heparin-precipitable fibrinogen was not demonstrated in the animals before or after the administration of globulin. Studies of total fibrinogen levels before and after globulin administration are being carried out at the present time.

It is difficult to compare the results of these studies with those obtained by other workers. This is true because of the varying amounts and types of foreign proteins used, the method and number of times of administration, and, in the present studies, the use of endotoxin or Liquoid in conjunction with the administration of globulin.

More and Waugh,[19] for example, subjected rabbits to unilateral nephrectomy followed by two intravenous injections of bovine gamma globulin in a dosage of 1 gm. per kg. of body weight. The injections were given 12 days apart, and the animals were killed 7 days following the last injection. In 77 per cent of these animals there were renal glomerular lesions which consisted of endothelial proliferation. In some animals the presence of "homogeneous acidophilic protein material" in some of the glomerular capillaries was observed. The glomerular lesions were reported as minimal in 22 per cent and as definite, marked proliferation in 55 per cent.

In other studies by More and McLean,[17] rabbits were given from one to eight intravenous injections of horse serum in amounts of 10 cc. per kg. at 18 day intervals. They reported no increased incidence of renal lesions in these animals as compared with controls given no horse serum. Hawn and Janeway[10] reported that 63 per cent of rabbits given intravenous bovine gamma globulin developed marked glomerular capillary endothelial proliferation, with droplets of acidophilic material in the capillaries.

Similar difficulties arise in an attempt to compare the inflammatory vascular lesions of the heart which were observed in the present study with those reported previously. Hopps and Wissler[14] gave rabbits two intravenous injections of horse serum in amounts of 10 cc. per kg. at an interval of 19 days. The rabbits were sacrificed 7 days after the last injection of serum. Of a group of 16 rabbits, they reported that 69 per

cent showed the presence of coronary arterial lesions resembling polyarteritis. These lesions were described as containing fibrinoid, and associated with a mononuclear cellular reaction.

Lesions in organs other than the heart and kidneys have been reported infrequently in animals given foreign proteins. Hopps and Wissler[14] described arterial lesions of the lungs, spleen, liver, kidney, and other organs in one rabbit of a group of 16 which were given horse serum intravenously. In the present studies, lesions of the lungs, spleen, and liver were observed commonly.

The renal lesions observed in the present experiments are similar to those of "focal" or "embolic" glomerulonephritis in humans, and in some respects resemble the changes of acute proliferative glomerulonephritis. The focal glomerular accumulation of large amounts of fibrinoid, with obliteration of capillary loops, is similar also to the lesion of diabetic glomerulosclerosis, but no hyaline material was observed in the walls of the afferent arterioles. The presence of fibrinoid material in the larger renal arteries, and its association with an inflammatory reaction in the coronary arterial walls are features similar to the lesions of polyarteritis.

These variations in the extent and localization of fibrinoid, and the presence or absence of an inflammatory reaction, are similar to the variations and overlapping of the lesions of the systemic fibrinoid diseases in man.[3] It is possible that changes in serum proteins, either in amounts or types, may influence the deposition of fibrinoid in certain sites.

## Summary

Hybrid albino rabbits were given a daily subcutaneous injection of 0.5 cc. of a 10 per cent solution of bovine gamma globulin for 6 days. Seventy-two hours after the last injection of globulin the animals were given a single intravenous injection of Gram-negative endotoxin or sodium polyanetholsulfonate (Liquoid). Those which did not die following the administration of toxin or Liquoid were sacrificed 48 hours later.

Fibrinoid lesions involving the heart, lungs, spleen, liver, and kidneys were observed in 85 per cent of the animals given endotoxin and in 61 per cent of those given Liquoid. The coronary arterial lesions were associated with an inflammatory reaction which resembled the changes of polyarteritis. The renal fibrinoid was deposited in a focal manner in scattered glomeruli and in the media of the large renal arteries. There was also a pronounced glomerular endothelial proliferation which resembled the changes of acute proliferative glomerulonephritis.

Serial paper electrophoretic studies of serum proteins were performed on 77 animals. These showed a reversal of the albumin-globulin ratio due to an increased amount of globulin and the presence of a para-beta globulin which migrated between the beta and gamma globulin fractions. Although no correlation could be obtained between changes in serum proteins and incidence of fibrinoid lesions, it is possible that the alterations in the serum proteins may have influenced the sites and amounts of fibrinoid formation or deposition.

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[ *Illustrations follow* ]

## LEGENDS FOR FIGURES

All sections were stained with hematoxylin and eosin unless otherwise specified.

FIG. 1. Renal glomerulus showing massive focal fibrinoid deposition in the upper half of the capillary loops while the lower loops appear relatively normal. The lesion resembles "focal glomerulonephritis" in humans. Rabbit given 500 μg. of *Escherichia coli* endotoxin 72 hours after a series of injections of bovine globulin. The animal died 13 hours later. × 350.

FIG. 2. Renal artery showing extensive fibrinoid deposition in the media and perivascular space with perivascular edema and minimal mononuclear cellular reaction. Rabbit given an injection of meningococcal endotoxin 72 hours after a series of injections of bovine globulin. The animal died 24 hours later. × 50.

FIG. 3. Interlobular artery from the same kidney as the artery in Figure 2. There is extensive medial deposition of fibrinoid material with associated hemorrhage, perivascular edema, and moderate numbers of mononuclear cells. × 250.

FIG. 4. Section from the kidney of a rabbit given 10 mg. of Liquoid 72 hours after a series of injections of bovine globulin. The animal died 14 hours later. There is a marked increase in the cellularity of the glomeruli. No fibrinoid material is visible. Movat[24] trichrome stain. × 65.

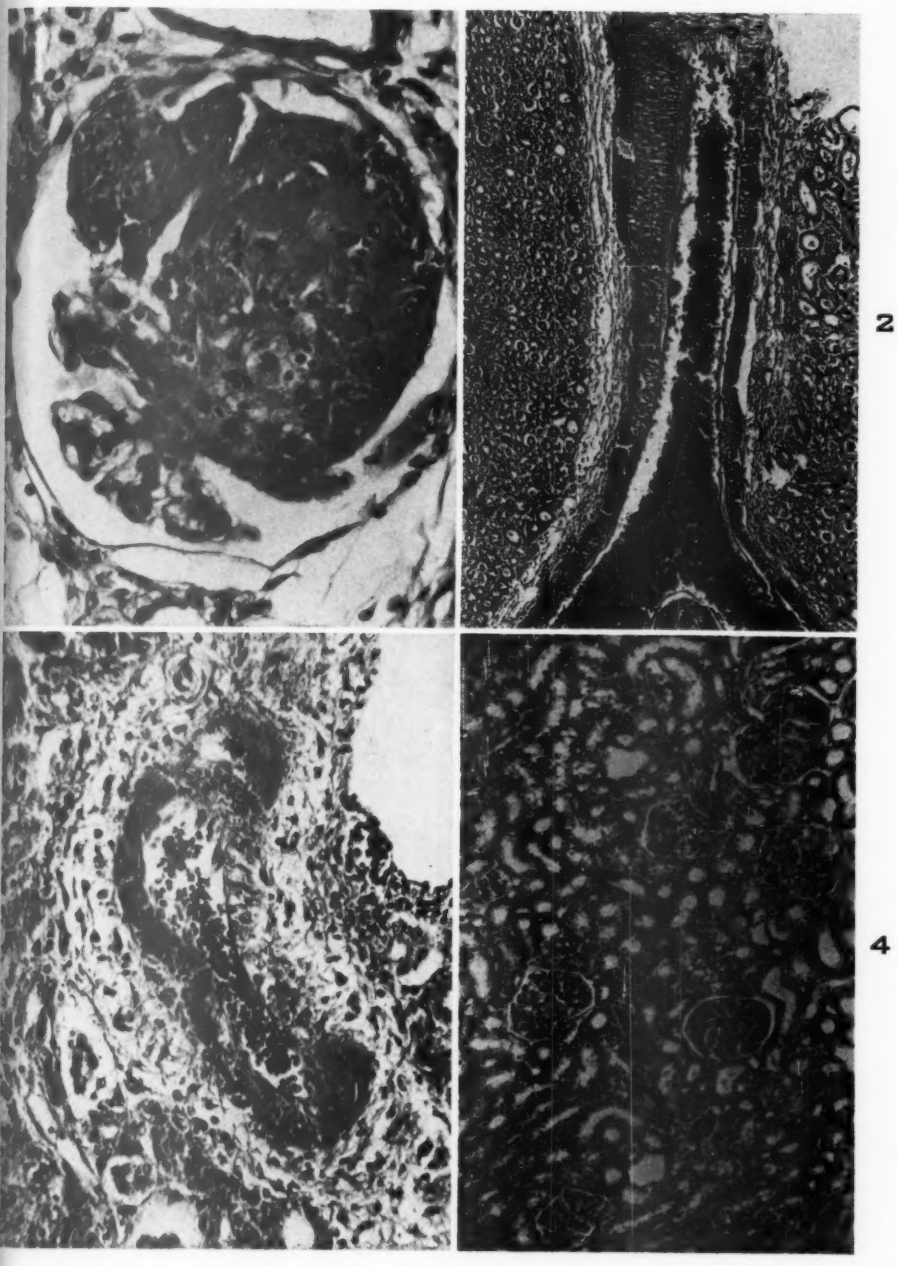

FIG. 5. Intramural coronary artery showing a marked perivascular infiltration by mononuclear cells with extension of the inflammatory process into the cardiac muscle. Rabbit given 10 mg. of Liquoid 72 hours after a series of injections of bovine globulin and sacrificed 48 hours later. × 250.

FIG. 6. Intramural coronary artery in a papillary muscle showing medial deposition of fibrinoid material and a marked mononuclear and heterophil cellular reaction about the artery. Rabbit given 10 mg. of Liquoid 72 hours after a series of injections of bovine globulin and sacrificed 48 hours later. × 250.

FIG. 7. Intramural coronary artery showing small, irregular, and subendothelial accumulations of fibrinoid material. There is a moderate cellular reaction about the fibrinoid material in the lower vessel. Rabbit given 500 μg. of *E. coli* endotoxin 72 hours after a series of injections of bovine globulin and sacrificed 48 hours later. Movat trichrome stain. × 350.

FIG. 8. Section from the liver of a rabbit given 500 μg. of *E. coli* endotoxin 72 hours after a series of injections of bovine globulin and sacrificed 48 hours later. There is extensive ischemic necrosis of the parenchyma, hemorrhage into the parenchyma, and surrounding zones of reaction. Movat trichrome stain. × 25.

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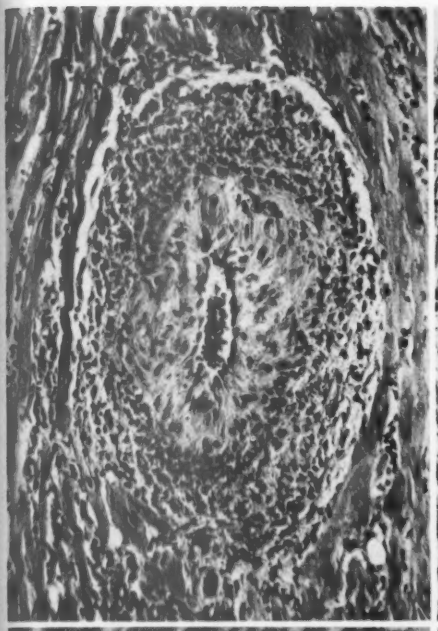

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# CYTOPATHOLOGIC CHANGES IN TISSUE CULTURES OF HUMAN AMNIONIC CELLS INFECTED WITH POLIOMYELITIS, COXSACKIE, AND ECHO VIRUSES *

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Tissue cultures of renal cells of monkeys and of certain human normal and malignant cells are widely used today for the cultivation of poliomyelitis, Coxsackie, and other viruses. During the past year, cultures of trypsinized human amnionic cells, originally introduced by Zitcer *et al.*,[1] have been used continuously by us for virus studies. The present paper gives a short description of some of the morphologic changes observed in fixed and stained cultures of amnionic cells following infections with poliomyelitis, Coxsackie, and ECHO viruses.

## Material and Methods

### *Tissue Cultures*

Cells obtained after tryptic digestion of human amnion, stripped from placentas after birth, were cultured on small pieces of microscopic slides, 6 by 25 mm., inside rubber-stoppered test tubes which were kept slightly slanted in a stationary position. A number of experiments also were carried out using cells grown directly on the test tube wall. In the latter case the collodion technique, described by Enders and Peebles,[2] was used to peel the cells off the glass. The medium used for planting the cells contained 0.5 per cent lactalbumin hydrolysate in Hanks's solution to which 20 per cent calf serum was added. After 2 days, fresh lactalbumin medium containing 2 per cent calf serum and 0.5 per cent chick embryo extract was added. When confluent growth had been obtained, the cultures on glass were transferred to fresh tubes with lactalbumin medium from which chick embryo extract was omitted and Earle's solution substituted for that of Hanks. The latter medium was employed also when cells grown directly on glass were exposed to virus infection. All media contained 100 units of penicillin, 100 μg. of streptomycin, 25 μg. of tetracycline, and 40 units of mycostatin per ml. Cultures from 17 different amnions were studied.

### *Virus Strains and Infection*

Most experiments on poliomyelitis infection were carried out with the type II $MEF_1$ strain. The cytopathogenic effects of the Mahoney

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and Saukett strains as representatives of types I and III and of two locally isolated type I strains were examined also.

To study the effects of infections with Coxsackie and ECHO viruses two locally isolated B3 strains, one A9 strain, and types 1, 3, 5, 6, and 7 of ECHO viruses were used.* All strains, after a number of passages in amnionic cells, were titrated and kept as frozen tissue culture fluids at −20° C. Their $TCID_{50}$† ranged between $10^{6.0}$ to $10^{8.0}$ per ml. These fluids were added in amounts of 0.1 ml. to batches of 20 to 40 tissue cultures as described above. After further incubation at 36° C., 2 to 4 cultures were removed at intervals of 2 hours up to 16 hours and after 24, 32, and 48 hours. The medium was discarded, and the cultures washed in Earle's solution and fixed in Helly's fluid. Staining was carried out with hematoxylin and eosin, Heidenhain's iron hematoxylin, or Giemsa's stain. Some preparations were stained with Harris' hematoxylin and eosin Y according to Reissig *et al.*[8] Non-infected cultures were fixed and stained at the same time.

## Results

### *Poliomyelitis Virus*

Cultures of poliomyelitis virus exhibited no pathologic changes until 6 hours after infection. At that time a few nuclei which were still normal in size and shape showed one or several straight, sharply delineated, colorless bands which sometimes crossed the whole nuclear substance (Fig. 1). Their borders stained darkly with hematoxylin. In addition, shorter or longer fine dark lines appeared which might be the first indications of wrinkling of the nuclear membrane (Fig. 2). The nuclear chromatin outside these bands and lines showed normal distribution and the nucleoli were clearly visible.

Eight hours after infection many nuclei had lost their round, smooth surface and showed shrinkage, indentation, and wrinkling of the membrane to various degrees. At that stage it frequently became difficult to recognize the nucleoli. Still later, the nuclei became pyknotic and appeared as dark, sharply angled, irregular masses which sometimes were located eccentrically. Eosinophilic cytoplasm was an early manifestation in cells which exhibited nuclear changes.

Eight hours after infection, the cells which had grown as a continuous sheet began to separate from each other. This change was still

* The poliomyelitis viruses were originally obtained from Dr. P. Lepine, Paris, France; the ECHO virus strains, from Dr. J. L. Melnick, New Haven, Conn. The Coxsackie viruses were identified with the aid of antisera obtained from Dr. G. Dalldorf, Albany, N.Y.

† Final limiting viral dilution which would initiate infection in 50 per cent of the cultures inoculated.

more pronounced at 10 hours. The cells frequently remained attached to their neighbors by thin cytoplasmic strands and spikes. Some of them were straight and extended over a considerable distance while others showed dendritic ramifications with slight swellings at their ends (Fig. 3). At the same time spherical cytoplasmic protrusions appeared, occasionally around the entire circumference of the cells. Portions of these protrusions and cytoplasmic strands seemed to detach themselves from the cell body and were found with increasing frequency as free-lying particles between the cells.

In a number of cells 12 to 14 hours after infection, a denser, more eosinophilic endoplasm, and a paler, generally very small rim of basophilic ectoplasm could be observed. However, this separation was not very pronounced. Another charactertistic change about 12 hours after infection was the appearance of granules in the cytoplasm of a number of cells which stained dark blue with hematoxylin or Giemsa's stain. These granules usually were found in the outer cell zone and sometimes in cytoplasmic processes.

Fourteen to 18 hours after infection, most cells were rounded and isolated. The cytoplasm showed vacuolization in some cells and a thin rim of basophilic ectoplasm in many others. The location of the nuclei was slightly eccentric, and in a small number of cells pronouncedly so (Fig. 4). It is remarkable that even at this advanced stage of infection an occasional mitotic figure was sometimes seen, indicating that some cells escaped infection for a long time.

Twenty-four to 48 hours after infection many cells had separated from the glass. Those cells remaining were shrunken, showed marked eosinophilia of the cytoplasm, and an eccentrically located pyknotic nucleus.

Whole cells or cell fragments sometimes were observed to lie within the cytoplasm of normal cells, due apparently to phagocytic activity. This activity seemed to increase when more degenerating cells were present, as it occurred in normal, older or infected cultures. It was not difficult, as a rule, to differentiate ingested cells or cell particles from viral inclusion bodies. True inclusion bodies were not found to occur, either in the cytoplasm or in the nucleus. Following the report of nuclear inclusions in infected renal cells of the monkey by Reissig *et al.*,[8] a special search was made for inclusions of this nature using the staining procedure recommended by these authors. In a few nuclei, eosinophilic bodies were found. The same bodies also were observed, however, in normal cells. The various poliomyelitis strains used in this study produced similar changes in all preparations examined.

*Coxsackie and ECHO Viruses*

The changes caused by viruses of both the Coxsackie and ECHO groups were in many respects similar to those described for poliomyelitis virus. The first cytologic changes were always observed in the nucleus, which showed wrinkling and folding of the membrane. Changes in the cytoplasm appeared later and showed a number of characteristics which served to distinguish them from those caused by poliomyelitis virus. These changes also served to differentiate the Coxsackie and ECHO virus strains from each other.

During the early stages of degeneration following Coxsackie virus infection the cells seemed to swell and showed a striking tendency to become round. This frequently gave them a balloon-like appearance. Very characteristically, a small number of cells showed elongation of the whole cell body. The cytoplasmic processes were fewer, not as thin as in cells infected with poliomyelitis virus, and very straight, rarely showing dendritic ramifications. Often prominent cone-like swellings appeared at the ends of the processes (Fig. 5).

The separation of the cytoplasm into a more eosinophilic homogeneous endoplasm and a paler ectoplasm was very pronounced. This separation began 6 to 8 hours after infection and was very marked at 10 hours (Fig. 7). The endoplasm in these cells formed a sharply delineated, round, oval, or sometimes angular body. The nucleus lay either in its center or, very characteristically, seemed to be pushed to the ectoplasm at the periphery of the cell. Generally, the nuclei showed a greater tendency to an eccentric position in the early stages of Coxsackie virus infection than after that caused by poliomyelitis virus. Active formation of cytoplasmic blebs, with the early appearance of many free-lying blebs between the cells, was seen. Dark-blue staining granules in the cytoplasm, like those described in cells infected with poliomyelitis virus, and vacuolization of the cytoplasm were observed in the later stages.

These changes were present after infection with two $B_3$ strains of Coxsackie virus. The findings after infection with one $A_9$ strain were similar. In the latter case, too, balloon-like enlargement of the cells, pronounced vacuolization of the cytoplasm, and a sharp separation of ectoplasm and endoplasm were observed. A larger number of Coxsackie strains will have to be examined before it can be stated that these changes are characteristic for all Coxsackie viruses cytopathogenic for human amnionic cells.

Whereas, after infection with poliomyelitis and Coxsackie viruses the first signs of infection were observed 6 hours later, it took some-

what longer to produce visible damage with the ECHO viruses. Infection with a high-titered suspension of type 7 ECHO virus (titer, $10^8$ per ml.) caused the first microscopic changes in the nucleus 8 hours later. Dissociation of the cells began at 12 hours and was pronounced at 14 hours. The isolated cells, which were sometimes large, characteristically showed no tendency to become round, but retained their irregular polygonal or triangular shape for a long time. Even 48 hours after infection a few cells of those still remaining on the glass had not completely rounded off. In the cytoplasm, vacuolization and rather coarse basophilic granules appeared at 14 hours. At that time a separation into a paler endoplasm and a more basophilic ectoplasm was seen in a number of large, irregularly shaped cells. Both zones, however, were not as sharply separated from each other as occurred after infection with Coxsackie virus. Straight and long cytoplasmic processes were present. These were similar to those described for infection with Coxsackie virus. Here, too, a number of cells showed extreme degrees of elongation and thinning of the whole cell body, including the nucleus. Most characteristic was the occurrence of completely normal appearing cells and cell groups side by side with severely damaged elements.

Infections with ECHO viruses, types 1, 3, 5, and 6, were carried out with suspensions of lower titer. In one experiment a type 7 virus suspension of lower titer was used also (titer, $10^{-6}$ per ml.). In these cases the first changes appeared 12 to 24 hours after infection (Figs. 8 and 9) and progression to complete degeneration took longer than described above. However, the same changes were observed. The picture produced by all ECHO strains examined was astonishingly uniform.

## Discussion

The reactions of different cell systems in tissue culture to virus infection are not uniform. Barski *et al.*,[4,5] working with human fibroblasts infected with poliomyelitis virus, found that the first reaction occurred in the cytoplasm in the form of a clearly delineated eosinophilic inclusion; nuclear changes appeared simultaneously or later. In poliomyelitis infected HeLa cells no specific cytoplasmic inclusions were described by Ackermann, Rabson, and Kurtz.[6] Harding *et al.*,[7] however, reported the occurrence of cytoplasmic inclusions in HeLa cells infected with a type II poliomyelitis virus. The first microscopic changes were observed in the nucleus. The renal cells of the monkey infected with poliomyelitis virus have been studied by a number of authors.[3,8-12] Dunnebacke,[12] in a study of such cells, HeLa cells, and human fetal cells, found the three cell types to behave similarly, the first changes appearing in the nucleus, and a cytoplasmic inclusion

appearing later. Similar observations in renal cells were made by Beale *et al.*[9] and by Reissig *et al.*,[3] who, as noted above, also observed the appearance of nuclear inclusions. The effect of poliomyelitis infection on human amnionic cells *in vitro* has recently been reported by Dunnebacke.[13] In many respects our observations are in agreement. No circumscribed cytoplasmic masses or inclusion bodies were observed by Dunnebacke, nor were they found in our preparations. Nuclear inclusions were not observed in our material, even when the staining procedure of Reissig *et al.* was utilized. It is concluded, therefore, that amnionic cells differ in this respect from the cell types described above. The first microscopic changes after poliomyelitis infection were always observed in the nucleus. Here our observations are not in complete agreement with those of Dunnebacke. In the majority of her preparations the first changes appeared in the nucleoli, which showed decreased staining with hematoxylin, and finally disappeared altogether. This was never observed by us. The nucleoli always remained clearly visible until the wrinkling of the nuclear membrane occurred. At that stage the nucleoli frequently became difficult to recognize. A possible explanation for the difference between our observations and those of Dunnebacke may be found in the use of different media during the cultivation and infection of the cells. The influence of different media on morphologic changes of cells *in vitro* following viral infection has recently been reported by Reissig *et al.*[14] This might also explain why growth curves of poliomyelitis virus in amnionic cultures carried out in our laboratory[15] showed an earlier release of virus from the cells than those reported by Dunnebacke.

In contrast to the numerous descriptions of the changes in tissue cultures following infection with poliomyelitis virus, few reports on the cytologic picture produced by Coxsackie and ECHO viruses in various cell types, and none on the reactions in amnionic cells, have so far appeared. Enders[16] stated that the effect of Coxsackie viruses in tissue culture was not to be distinguished with certainty from that caused by poliomyelitis virus although swelling and the development of "bizarre" forms were usually not as striking. The changes produced by the two Coxsackie $B_3$ and the $A_9$ strains in amnionic cells were, in our experience, characteristic and different from the changes produced by either poliomyelitis or ECHO viruses. The early rounded appearance of the cells, the straight cytoplasmic processes, and the sharp separation of ectoplasm and endoplasm facilitate the recognition of these viruses as a separate group. The changes occurring after infection with ECHO viruses seemed also sufficiently specific to separate them from those produced by viruses of the other two groups. The tendency of the

ECHO infected cells to retain a polygonal or triangular shape and the simultaneous occurrence of normal cells adjacent to severely damaged areas, probably due to unequal susceptibility of the cells, were characteristics of the five types of virus studied. The latter property has also been observed by Enders in cultures of monkey kidney after infection with undifferentiated viruses from human feces—viruses which, according to the description given, would today be included in the ECHO group. The prolonged persistence of normal appearing cells within plaques produced by ECHO virus in cultures of monkey kidney was reported by Hsiung and Melnick.[17] The plaques caused by orphan viruses in these cultures were found to differ considerably from those produced by Coxsackie and poliomyelitis viruses.

Specific cytopathologic changes caused by different viruses in tissue culture are not only of theoretical interest but may also be used for practical purposes. The ever growing number of serologic types among Coxsackie and ECHO viruses and their frequent presence in "normal" stool specimens make the immunologic identification of a virus isolated from feces a cumbersome procedure. It would be of value if cytopathologic differences in tissue culture could be used for a rough preliminary classification. The differences in plaque structure described by Hsiung and Melnick[17] for various viruses indicate that such possibilities exist. It seems possible from our results that instead of the uneconomical plaque technique, cytologic changes in infected test tube cultures could be used for differential diagnostic purposes. For this purpose it is necessary to examine the cultures before the appearance of the final stages of degeneration, which are usually of a non-specific character. While a diagnostic procedure based on cytopathologic differences will always need final serologic confirmation, it may be of similar value to the virologist as the use made by bacteriologists of the different structure of bacterial cultures for a rough, preliminary orientation. Additional staining techniques and cytochemical methods may reveal further cytologic differences. The ultimate criterion of the usefulness of this approach to a differential diagnosis of enteric viruses will depend on the experience gained from the examination of a larger number of freshly isolated strains.

## Summary

The morphologic changes observed in tissue cultures of human amnionic cells after infection with poliomyelitis, Coxsackie, and ECHO viruses are described. A number of differences in the reaction of the cells to infection with the three types of virus were observed. It is proposed that such differences may be of value in a preliminary differential diagnosis of the enteric virus group.

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[ *Illustrations follow* ]

## LEGENDS FOR FIGURES

All photomicrographs were taken of cultures stained with hematoxylin and eosin, with the exception of Figures 3 and 4 which were stained with Heidenhain's iron hematoxylin. Cultures used for Figures 1 to 7 were grown on pieces of microscopic slides; cultures used for Figures 8 and 9 were grown directly in the test tube and treated with collodion afterwards.

FIG. 1. Six hours after infection with poliomyelitis virus (titer, $10^{-7.5}$ per ml.). Nuclei show straight, sharply delineated, colorless bands crossing the nuclear substance. × 1,300.

FIG. 2. Same culture as that from which Figure 1 was taken. Nucleus shows fine dark lines. × 1,300.

FIG. 3. Ten hours after infection with poliomyelitis virus. Cytoplasmic processes show dendritic ramifications and slight swelling at their ends. × 370.

FIG. 4. Eighteen hours after infection with poliomyelitis virus. Isolated cells, some showing vacuolization of the cytoplasm, others showing fine processes and small ectoplasmic rim. All nuclei are pyknotic. × 450.

FIG. 5. Six hours after infection with Coxsackie virus $B_3$ (titer, $10^{-7.5}$ per ml.). Rounded cells show straight processes with cone-like swellings. Beginning separation of endoplasm and ectoplasm. × 450.

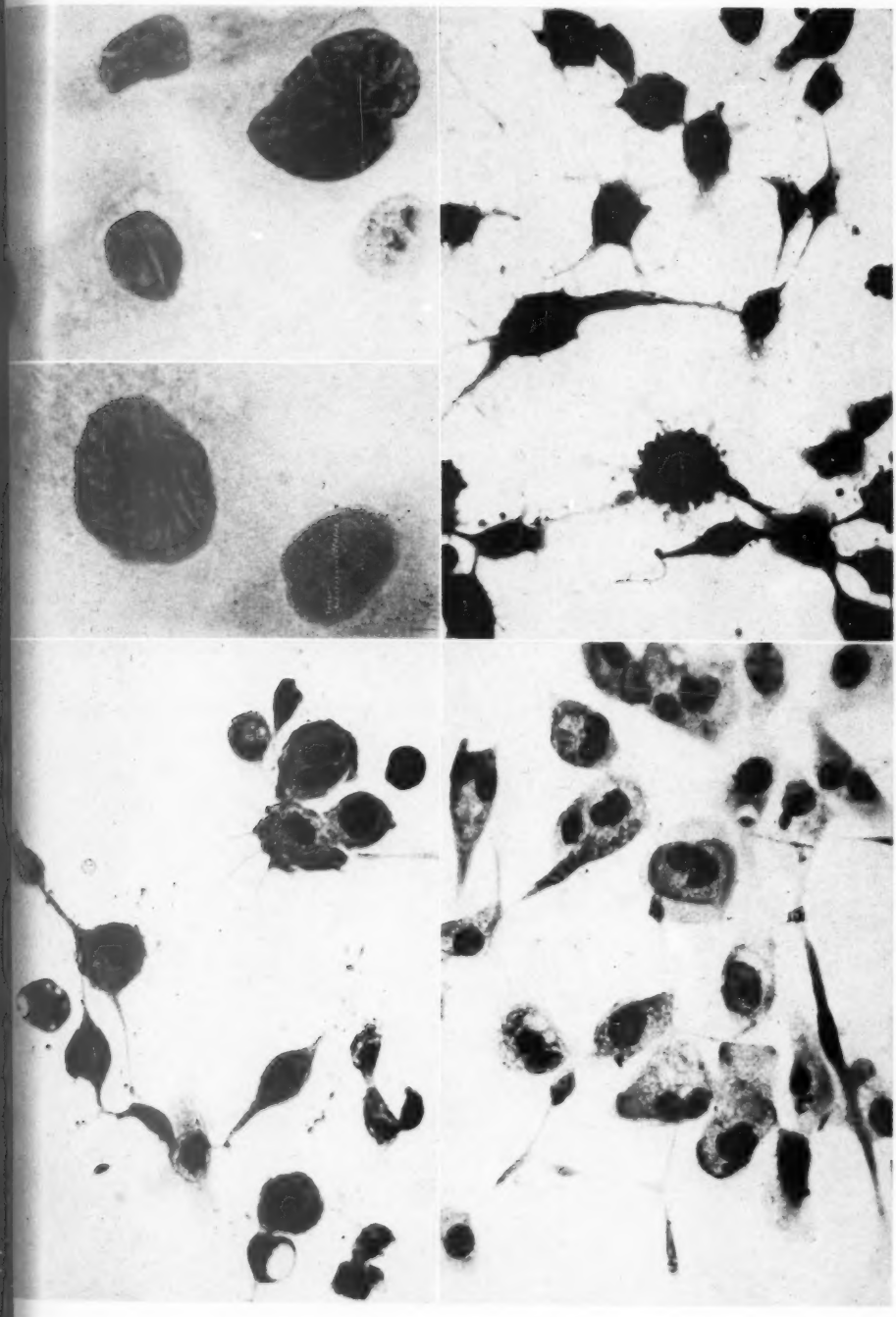

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FIG. 6. Eight hours after infection with Coxsackie virus $B_3$. Cells completely rounded show pronounced separation of endoplasm and ectoplasm. Many nuclei pushed to periphery. × 460.

FIG. 7. Ten hours after infection with Coxsackie virus $B_3$. Beginning shrinkage of cells with pyknosis of nuclei. One very large cell with characteristic cytoplasmic separation. × 500.

FIG. 8. Twenty-three hours after infection with type 7 ECHO virus (titer, $10^{-6.0}$ per ml.). Cells of irregular shape, some showing pyknotic nuclei, lying side by side with unaltered cells. × 500.

FIG. 9. Same culture as that from which Figure 8 was taken. Large swollen cells of irregular shape. Two cells show thinning and elongation. Some cells still unaltered. × 500.

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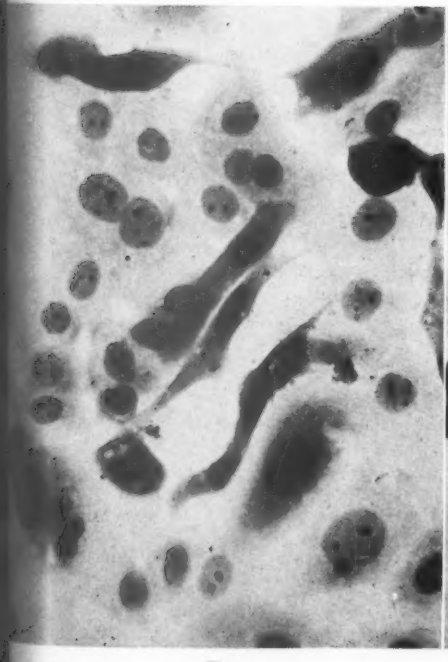

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## STUDIES ON CALCIFICATION

# I. The Effect of Inhibition of Enzyme Activity on Developing Bone and Dentin *

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Sodium fluoride at a physiologic pH has been observed to produce an inhibitory effect *in vitro* upon an esterase found in the matrices of calcifying bone and dentin.[1] Based upon this observation, toxic, but not lethal, doses of sodium fluoride were administered to a series of immature hamsters in an attempt to produce an inhibition of enzyme activity *in vivo*.

In addition to studies on the distribution of enzymes, a number of specific and semi-specific staining procedures were employed in an attempt to elucidate, in part, the pathogenesis of the toxic effects of fluoride upon calcifying tissues. The hamster was selected as the experimental animal on the basis of preliminary studies which revealed a higher esterase activity of its bones as compared with the rat and mouse.

## Methods

Seven litters of hamsters, 2 to 3 days old, each weighing approximately 3 gm., were given injections of sodium fluoride solutions ranging in molarity from 0.02 to 0.05. One to three intraperitoneal injections were given daily for 3 successive days.†

The characteristic changes were obtained following injections of 0.1 ml. of 0.05 M sodium fluoride twice a day (morning and afternoon) for the 3-day period. Controls consisted of untreated animals and animals injected with saline solution.

The animals were sacrificed by decapitation 1 to 2 hours after the final injection, and the heads and knee joints were frozen in isopentane cooled by liquid nitrogen (−160° C.). The tissues were dehydrated under vacuum (−30° C.), infiltrated and embedded in paraffin (U.S.P., m.p. 56° C.), sectioned serially at 10 $\mu$, and dry-mounted. Sections from control and fluoride-treated animals were mounted on the same slide.

Representative sections were stained with hematoxylin and eosin as well as metachromatic stains (toluidine blue and celestin blue).

* Received for publication, May 6, 1957.

† In preliminary experiments some of the solution escaped following withdrawal of the needle. It was found that this could be minimized by injecting through a fold of abdominal skin held between the forefinger and thumb.

Methods for the demonstration of protein and glycol groups included the dinitrofluorobenzene (DNFB), ninhydrin-Schiff,[2] and the periodic acid-Schiff (PAS) procedures.

Esters of Naphthol AS-MX* (the 3.5 xylidide of 2-hydroxy, 3-naphthoic acid) were used as substrates. In addition to Naphthol AS-MX acetate,[1] a more complex fatty acid ester of another highly chromogenic naphthol was prepared, Naphthol AS-LT isobutyrate, $C_{23}H_{23}O_4N$, M.W. 377.4. *Calculated:* C, 73.2%; H, 6.14%; N, 3.72%; *Found:*† C, 73.03%; H, 6.25%; N, 3.62%; m.p. 121° C.

The substrate (MX-acetate) was dissolved in 0.5 ml. of dimethylformamide (DMF) followed by distilled water and pH 7.1 Tris buffer to make a total volume of 50 ml. The pH of the buffer must be adjusted as carefully as possible. (It is of interest to note that phosphate buffer has a very marked inhibitory effect.) One ml. of DMF was used to dissolve the LT-isobutyrate. Twenty mg. of diazotized amino-azotoluene (Garnet GBC salt‡) were added and the mixture shaken and filtered through rapid filtering paper into a Coplin jar. Sections were incubated for 30 to 40 minutes. Many commercial diazonium salts are unsatisfactory since they are zinc compounds, and zinc has been observed to be a powerful inhibitor of the reaction.[1]

Some sections were stained to demonstrate alkaline phosphatase using an azo-coupling procedure employing a new substrate, Naphthol AS-MX phosphate.[3] Blue shades are produced with fast blue salt BBN,‡ while red and violet shades are obtained with fast red RC‡ and red-violet LB salts, respectively. With the frozen-dried tissue the incubation time is only 20 to 25 minutes. Although alpha-naphthyl phosphate does not give as sharp results as the AS-MX phosphate, the incubation time is very short (5 to 10 minutes). In addition, sections were stained using both esterase and phosphatase procedures. The sections were first incubated in the esterase substrate, after which they were rinsed in distilled water and then transferred to the phosphatase substrate solution.

## Observations

### *Hematoxylin and Eosin, Protein-Group, and Other Methods*

With hematoxylin and eosin there was no difference in the staining reaction of bone and dentin of fluoride-treated and control animals. In some of the fluoride-treated hamsters, there was a decrease in the

* Naphthols furnished through the courtesy of the Pfister Chemical Works, Ridgefield, N.J.

† Microanalyses done by Dr. W. Alford and group, U.S. Public Health Service.

‡ General Dyestuff Corp., New York, N.Y.

cellularity of the bone marrow as well as a decreased thickness of cortical bone. The protein-group, metachromatic, and PAS methods likewise revealed no difference in structure of bone or dentin. It may be of interest to note that the pre-dentin of normal frozen-dried material stained intensely with the protein-group and PAS methods. Details of PAS reactivity of frozen-dried dentin and bone have been described previously.[4,5]

*Enzyme Methods*

The reaction for demonstration of alkaline phosphatase revealed no appreciable difference between fluoride-treated and control animals. However, with the esterase procedure (MX-acetate or LT-isobutyrate) striking differences were observed between the two groups. The acetate was more rapidly hydrolyzed than the isobutyrate, and the following findings are related to its use.

In agreement with results of a previous study,[1] the esterase reaction of normal bone and dentin was extracellular, and portions of the matrix stained a brilliant shade of red. The earliest zone of matrix adjacent to osteoblasts was not reactive, while next to this unstained area the matrix reacted intensely. In some areas a granular distribution of staining was observed (Fig. 1). As previously observed, the central calcified portion of older spicules showed no activity. By analogy, pre-dentin showed no activity, an intermediate zone adjacent to calcified dentin reacted intensely, while older dentin stained slightly or not at all (Fig. 2). The cementum side of the incisor exhibited a zone of activity in the area of cementum apposition. The pattern of staining observed in dentin was essentially the same as in bone except that the zones were wider in the former. Incisor dentin showed numerous stained globules corresponding, in part, to globular dentin as seen with hematoxylin (Fig. 2). Reactive globules sometimes were seen in the odontoblasts, although this was not a consistent finding.

Control sections stained to demonstrate both esterase and phosphatase revealed not only a striking contrast between reactive elements, but clearly indicated a mutually exclusive distribution of each enzyme (Fig. 3).

The controls of the knee joints also stained intensely with the AS procedure, with the greatest reaction observed adjacent to the hypertrophic zone of cartilage cells (Figs. 4 and 5). Quantitative studies of extracts of the frozen-dried knee joint also revealed considerable esterase activity.[6]

The activity of bone and dentin in all fluoride treated animals was greatly diminished. Some areas of bone and dentin which normally

react intensely were totally unstained. It is interesting to note that the esterase activity of soft tissue structures in the head did not appear to be inhibited in the fluoride treated animals. These tissues included oral, lingual, and sinus epithelium, ducts of salivary glands, and nasal mucous glands.

## Discussion

Of interest is the fact that calcifying matrices will catalyze the hydrolysis of esters of beta-hydroxy-naphthoic acid anilides. As previously observed, esters of other naphthols were not hydrolyzed.[1] It was found also that, like fluoride, zinc, copper, and cadmium had a profound inhibitory effect *in vitro* upon the reaction of bone, yet produced no inhibition of the reaction of soft tissue. In the present work, systemically administered fluoride likewise was capable of producing inhibition of enzyme activity of bone.

On the other hand, alkyl fluorophosphates, which had no visible effect upon the reaction of bone and dentin, had a marked inhibitory effect on all soft tissue activity.[1] This characteristic difference of inhibitor effects would suggest the presence of a different enzyme in calcifying matrices as contrasted with that of soft tissues, although both are capable of hydrolyzing the same substrates. In a previous histochemical study, it was found that aminopeptidase was similarly inhibited by the same metals, while unaffected by diisopropyl fluorophosphate (DFP).[7] Benditt[8] also found that Naphthol-AS acetate was hydrolyzed by certain proteolytic enzymes. The possibility that the activity of bone and dentin is represented by a proteolytic enzyme should be investigated.

The suggestion that fluoride inhibits enzymes related to calcification has been considered by several authors.[9] However, it has been observed that alkaline phosphatase of calcifying structures is not particularly sensitive (during initial stages of administration) to fluoride. Smith and Lantz[10] concluded that fluorosis of tooth and bone structure is not mediated by phosphatase inhibition. Glock[11] suggested that this would indicate the presence of some other enzyme system, possibly one operating best at physiologic pH.

Leicester[9] reviewed the findings of Roche and Mourgue[12] and others who have suggested the rôle of a proteolytic enzyme in calcification. He mentioned the possibility of liberation of a proteolytic enzyme from ameloblasts, which would result in loss of organic matter before the final stages of calcification of enamel. By analogy, the presence of a proteolytic enzyme in bone and dentin may be related to the lysis of matrix concomitant with calcification.

## Summary

It was found that calcifying bone and dentin of the hamster would catalyze the hydrolysis of esters of complex hydroxy-naphthoic acid anilides. This reaction was greatly or completely inhibited in animals which received toxic, but not lethal, dosages of sodium fluoride.

The distribution and intensity of the alkaline phosphatase reaction were unchanged in the fluoride-treated animals. A series of specific and semi-specific staining reactions likewise revealed no histologic difference between the matrices of bone and dentin of fluoride-treated and control hamsters.

The simultaneous use of esterase and phosphatase methods on sections of developing bone may be of value because of their mutually exclusive distribution.

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[*Illustrations follow*]

## LEGENDS FOR FIGURES

FIG. 1. Esterase activity of matrix (intramembranous bone), showing granular distribution of activity. × 550.

FIG. 2. Cross section of incisor tooth showing esterase activity of zone adjacent to predentin. × 420.

FIG. 3. Esterase activity (red) of matrix of bone, and oral and lingual epithelium. Alkaline phosphatase activity (blue) (Naphthol AS-MX phosphate), of tissue adjacent to matrix of bone and of oral and lingual basement membrane. × 130.

FIGS. 4 and 5. Calcifying cartilage (knee joint), of control (Fig. 4) and fluoride-treated animal (Fig. 5). × 130.

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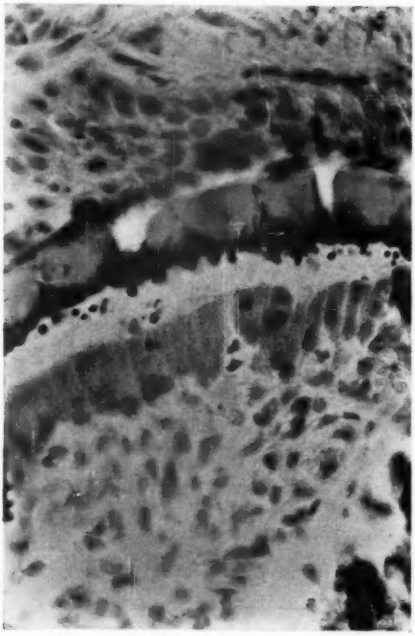

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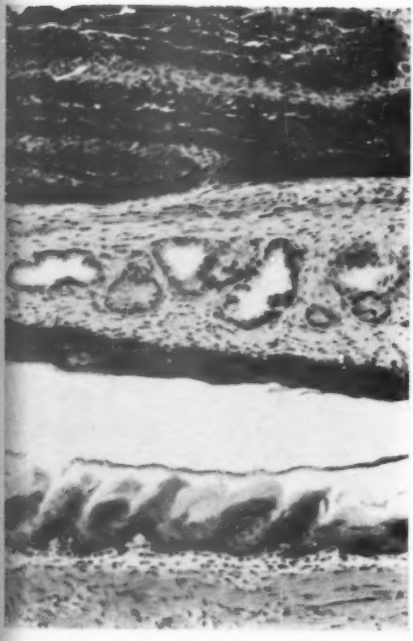

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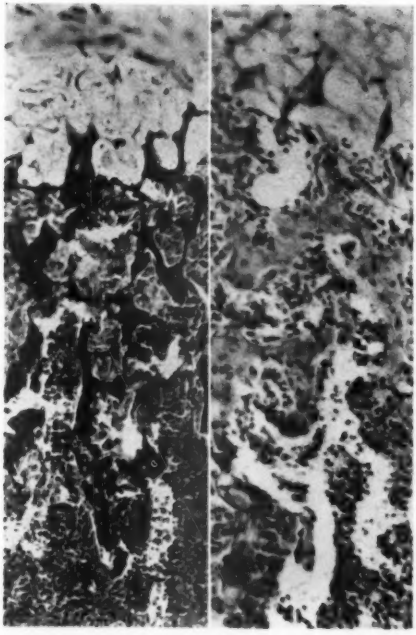

4 5

# CARTILAGE CHANGES IN PAPAIN-TREATED RABBITS *

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In a recent report, Thomas[1] stated that crude papain caused the collapse of ears when administered intravenously to young rabbits, whereas crystalline papain lacked this property. The histologic changes noted in the cartilaginous structures included a decrease in the amount of intercellular matrix and a loss of its hematoxophylic properties. This experimental procedure, aside from its significance as a method of assaying a new enzyme, would appear to provide an approach to the histochemical study of cartilage and to the functional differentiation of its constituents. The present investigation, employing histochemical methods applicable to the components of cartilage, was undertaken in an attempt to evaluate the histologic changes in greater detail and to clarify the mechanism of the papain effect.

## Methods

Young albino rabbits of the New Zealand strain, weighing less than 1 kg., were given 1 cc. of 2 per cent extract of crude papain in normal saline solution by ear vein. Punch specimens of the ear were taken for examination at approximately 30 minute intervals for 2 hours and less frequently thereafter for 24 hours. Necropsy specimens were obtained from animals sacrificed 7 hours and 1, 3, 9, and 22 days after injection. Specimens were fixed in Lillie's acetic alcohol formalin (AAF).[2]

Paraffin sections stained with hematoxylin and eosin were examined initially. In order to evaluate changes in specific constituents, additional sections were stained as follows: for polysaccharide, by the periodic acid-Schiff (PAS) procedure; for acid mucopolysaccharide, with azure A and with the alcian blue and Hale dialyzed iron techniques; for amino groups, with the ninhydrin Schiff method; and for elastica by the orcinol new fuchsin method of Fullmer and Lillie[3] and the direct Schiff stain. Staining procedures were used as given in detail by Lillie.[2] Azure A in 0.02 per cent concentration applied for 30 minutes was employed routinely in the demonstration of metachromasia.

* Received for publication, April 27, 1957.

† Fellow of the National Foundation for Infantile Paralysis.

## Results

The lesions observed in aural cartilage after the injection of crude papain fall into two general categories: (a) those seen in the first 3 hours, before collapse of the ear occurred; (b) those noted 4 hours or more following injection. One to 2 hours after injection the interlacunar septae appeared markedly increased in width and took a pale gray stain with hematoxylin and eosin (Fig. 2). At the same time, the chondrocytes underwent crenation so that empty spaces surrounded the shrunken cells. These appeared to consist largely of one or two pyknotic nuclei and a large vacuole. After 4 hours the septae returned to normal or even decreased in width while the chondrocytes re-expanded and filled the lacunae.

Sections stained with azure A at pH 2 demonstrated the concentration of metachromatic material in perilacunar areas in normal cartilage as described by Follis and Berthrong.[4] One to 2 hours after the injection of papain there was marked over-all increase in metachromatic material which was no longer concentrated around chondrocytes. Metachromasia was completely absent in specimens obtained 4 to 24 hours after injection (Figs. 4 to 6). Additional evidence confirming the loss of acid mucopolysaccharide 4 hours after injection was obtained with both the alcian blue and the Hale dialyzed iron stains.

Sulfation of the tissues according to the method of Kramer and Windrum[5] was attempted, by immersing the sections briefly in 20 per cent sulfuric acid prior to azure A staining. This pretreatment was found to impart considerable metachromatic staining to cartilage of specimens taken 24 hours after injections of papain. No such effect was evident in 4 hour specimens. Sections which were stained with eosin or with other acid dyes revealed a marked loss of acidophilia at 2 hours but a definite increase over normal after 4 hours. The ninhydrin Schiff and other methods for amino groups gave equivocal results on these sections.

As shown in Figures 7 to 9, the PAS-positive polysaccharide of chondrocytes was decreased and dispersed in granules in specimens taken at 4 hours. This confirms Thomas'[1] qualitatively similar finding. Digestibility in diastase identified this substance as glycogen. The decrease of glycogen was found to occur at about 4 hours and to persist through 24 hours. It was also noted in both biopsy and necropsy specimens that although metachromatic components disappeared from the matrix following papain, most of the PAS-positive material did not.

The direct Schiff stain using sulfite leukofuchsin without prior periodate oxidation is known to color rodent elastic fibers purple, presumably because of an aldehyde component peculiar to this order. The

elastic fibers of aural cartilage appeared disrupted and stained weakly or not at all with this method during the early post-papain phase, but they largely recovered after 4 hours (Figs. 10 to 12). The recovery coincided with the loss of metachromatic material as shown above. Papain failed to alter the staining reaction in adjacent arterial elastica, however, suggesting that the changes in elastic fibers of cartilage are possibly secondary to the other structural alterations. Moreover, with the orcinol new fuchsin procedure of Fullmer and Lillie[3] the elastic fibers, although fragmented and disrupted, continued to stain.

Within 7 hours after papain and for more than 24 hours thereafter the perichondrial collagen fibers throughout the body assumed a metachromatic, reddish purple color evident down to pH 3 (Figs. 13 and 14). Thin, beaded, similarly metachromatic fibrils and granules often were seen between the purple collagen fibers. At the same time perichondrial lymph vessels contained metachromatic, granular, globular or fibrillar material which tended to localize along the vessel wall. This substance stained purple with azure A at pH 2, as did normal cartilage matrix (Figs. 14 and 17). Masses of metachromatic granules and fibrils were noted between intercostal muscle bundles. Similar material could be seen in the cortical sinusoids of regional lymph nodes (Fig. 16) where in some instances it appeared to coat the lymphocytes.

Edema often developed in the dermis of the ear at any early time following papain, and in this area many coarse granules and fibrils filled the spaces between the collagen fibers. In specimens obtained later, a leukocytic infiltration occurred in this area. The granules and fibrils stained blue with azure A only above pH 4.5 (Fig. 15). The material proved to be acidophilic with eosin alone or with an eosin azure A mixture at pH 4.0. The metachromatic material in the interstitial spaces and/or lymphatics, on the other hand, stained weakly or not at all with eosin alone and was basophilic at pH 4.0 with the eosin-azure A mixture. Both groups of substances gave a pink to red color with the PAS method.

Decalcified sections made through the knee joint showed absence of metachromasia in the articular cartilage 1 day after injection. This contrasted sharply with the retention of intense metachromasia in the transforming cartilage of the primary spongiosa at the endochondral zone of ossification (Fig. 17). With the von Kossa and alizarin red S stains, undecalcified sections showed calcium in this area. In the intervening zone of hypertrophic chondrocytes aligned in linear fashion, between articular cartilage and primary spongiosa, the matrix remained moderately basophilic.

The recovery of the cartilage matrix following alterations induced

by papain has not been studied extensively, but limited observations are in agreement with those of Thomas[1] that there was partial restoration of metachromasia at 3 days. Nine days following injection, recovery remained incomplete. This was evident in the tracheal cartilage which revealed perilacunar metachromatic rings separated by more lightly stained, orthochromatic mid-septal matrix. The cartilage appeared normal at 22 days.

Crystalline papain* caused moderate crenation of chondrocytes and disruption of elastic fibers and some increase in the metachromasia observed at pH 2, but no loss of localization or escape of metachromatic material.

## Discussion

The present findings in cartilage agree in general with those of Thomas[1] for comparable time intervals, i.e., 4 hours or more after injection. The specimens procured early in this study show loss of perilacunar localization of metachromatic material, swelling of interlacunar septae, and crenation of chondrocytes, all of which point to release of slowly diffusible and osmotically active substances. The markedly increased metachromasia at this time indicates release of previously bound, strongly acid groups. Sulfate is the only known group in cartilage sufficiently acid to react with azure A at pH 2.0. The concurrent appearance of slowly diffusible substances and sulfate groups strongly suggests that the acid radical served originally to bind the released component to matrix. The freed acid constituent is seen to have diffused into perichondrial interstitial spaces and lymph vessels coincidentally with loss of cartilage metachromasia. The slow diffusibility with retention in the perichondrial lymphatics 20 hours after separation from the cartilage, and the tendency to form large aggregates insoluble in the fixative solution, indicate that the material is a polymer. It is also judged to be a sulfated polysaccharide on the basis of its metachromasia at pH 2 to 3 and positive PAS reaction. In their comprehensive review on metachromasia, Schubert and Hamerman[6] associated this property with high molecular weight. The evidence then favors the assumption that crude papain acts to liberate the sulfated polysaccharide, chondroitin sulfate, from cartilage. The known proteolytic properties of papain suggest that this effect comes about through degradation of matrix protein. However, the histochemical evidence indicating loss of matrix protein is equivocal. A recent demonstration of elastase activity in crude papain similar to that in pancreatin[7] would implicate this factor in the changes in elastic cartilage, except that pancreatic extracts in our hands fail to cause collapse of rabbit ears.

* Worthington papain 2× crystallized, Worthington Biochemical Corp., Freehold, N.J.

If the orthochromatic débris observed in the edematous corium also derives from cartilage chondroitin, it has through some modification lost its basophilic properties and become acidophilic. A combination of the acid cartilage constituent with protein in the serous exudate would account for both of these changes. Indeed, the marked eosinophilia of the material supports this possibility. Such a modification, also thought to occur when the cartilage-derived component enters the blood stream, will be discussed in a succeeding paper.[8] At least some of the material leaking from cartilage demonstrates a distinct affinity for collagen in its tendency to impart metachromasia to the perichondrial collagen fibers. Apparently then, the acid polysaccharide retains a capacity to react with certain proteins in blood and serous exudate with loss of basophilia, and on collagen fibers with retained metachromasia. This observation indicates that the complete escape of sulfated polysaccharide from cartilage depends on alteration or removal of protein sites in the matrix capable of binding the polysaccharide, rather than on degradation of the latter.

It is of interest that the matrix retains strong PAS staining and hence considerable polysaccharide after papain has removed the metachromasia. This may be related to the findings of Joel *et al.*,[9] who assumed the presence of a neutral hexosamine polysaccharide in cartilage and provided evidence that the proportion of this material to the acid polysaccharide increases with age. Papain-treated rabbits in which these substances are resolved would provide, then, an initial step in an attempt to characterize the non-sulfated cartilage polysaccharide.

The finding that the primary spongiosa cartilage, at the endochondral line of ossification, retains metachromasia after papain differentiates the chondroitin in this area from that in other cartilage. This difference undoubtedly relates to the ossification process, perhaps more specifically to the calcification known to occur at this site.

## Summary

Changes in the cartilage of rabbit ears 1 to 2 hours after intravenous injections of crude papain include dispersal of metachromasia, increase in metachromasia with decrease of acidophilia, swelling of interlacunar septae, and crenation of chondrocytes. Decrease in glycogen of chondrocytes and disruption of elastic fibers also occur.

Subsequent changes about 4 hours after the administration of crude papain include complete loss of metachromasia of matrix, increased acidophilia, and retained PAS reaction, as well as the return to normal of interlacunar septae, elastic fibrils, and chondrocytes. At the same time metachromatic granules and fibrils are found in perichondrial

interstitial spaces, lymph vessels, and regional lymph nodes; and perichondrial collagen fibers stain metachromatically. Orthochromatic granules and fibrils are seen in edematous areas in the corium of the ear and in blood vessels.

Papain fails to alter metachromasia in cartilage of the primary spongiosa at the line of endochondral ossification.

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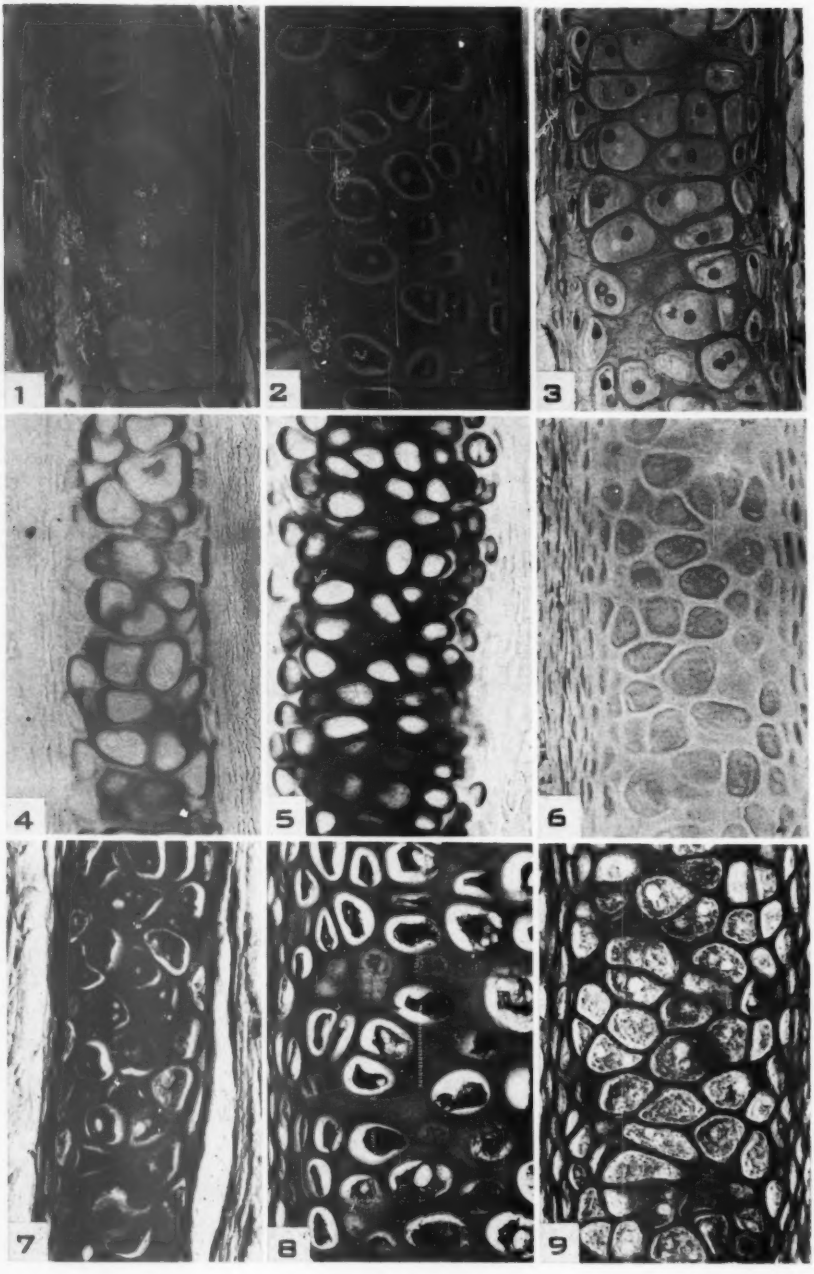
## LEGENDS FOR FIGURES

FIGS. 1 to 3. Biopsy specimens of aural cartilage: (Fig. 1) before, (Fig. 2) 2 hours after, (Fig. 3) 22 hours after papain, showing swelling of septae with crenation of chondrocytes at 2 hours and recovery but increased eosinophilia at 22 hours. Hematoxylin and eosin stain. × 260.

FIGS. 4 to 6. As in Figures 1 to 3, showing early increase and dispersal, and subsequent loss of metachromasia. Azure A stain at pH 2.0.

FIGS. 7 to 9. Biopsy specimens of the ear: (Fig. 7) before, (Fig. 8) 2 hours after, (Fig. 9) 4 hours after papain, showing decreased glycogen content of chondrocytes and retained matrix staining at 4 hours. Periodic acid-Schiff (PAS) stain. × 260.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FIGS. 10 to 12. As in Figures 1 to 3, showing early loss of staining of elastic fibers and subsequent recovery. Direct Schiff stain without periodic oxidation. × 260.

FIGS. 13 to 15. (Figs. 13 and 14): Tracheal mucosa of control rabbit and of rabbit 24 hours post-papain. There are metachromatic collagen fibers and also a metachromatic fibrillar material between collagen fibers and in lymph spaces after papain. Azure A pH 4.5. × 130. (Fig. 15): Edematous area in corium of the ear, showing granular material between collagen fibers. Azure A pH 6. × 415.

FIGS. 16 to 18. (Fig. 16): Lymph node of 24 hour post-papain rabbit showing metachromatic granules and fibrils in adjacent lymphatic and in cortical sinusoid. Azure A pH 4. × 130. (Fig. 17): Perichondrial lymph vessel 22 hours after papain, containing metachromatic granules and fibrils. Azure A pH 2. × 260. (Fig. 18): Line of endochondral ossification of upper tibia, showing loss of metachromasia in epiphyseal cartilage (above), basophilia in area of hypertrophic chondrocytes and retained metachromasia in spicules of mineralized cartilage (below). Azure A pH 4. × 260.

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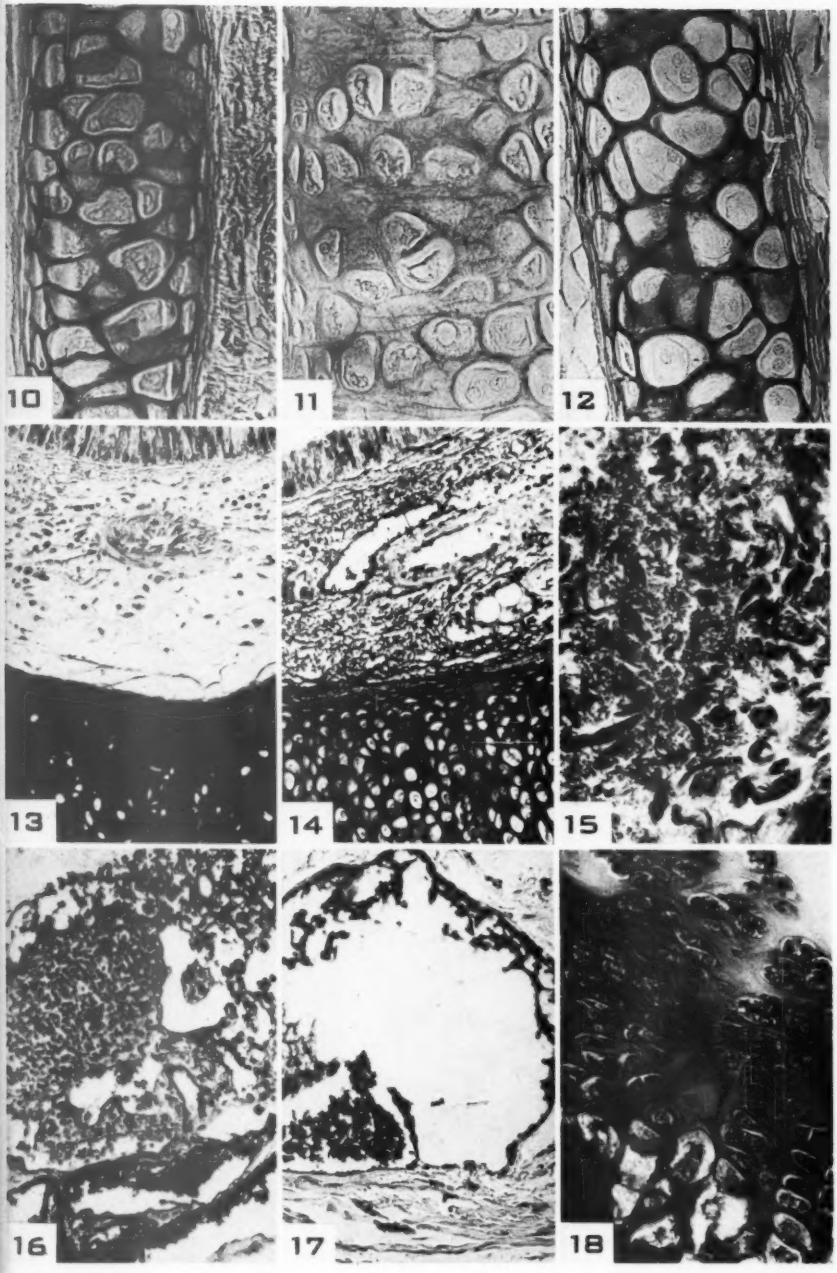
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